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The Effect of Selective Catheterisation of the Renal Artery Upon Renal Function in the Dog.

By

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Refinement of techniques for catheterisation of blood vessels has made it possible to perform selective catheterisation of several different organs. In this connection, however, LINDBLOM (1957) and WICKBOM and BARTLEY (1957) have demonstrated that a catheter introduced into the brachial artery can initiate vasoconstriction about the tip of the catheter. Peripheral ischaemia, it appears, is a possible sequela to catheterisation of medium-sized arteries.

Selective catheterisation of the renal artery of two dogs and two cats was used by LINDELL and OHLIN (1957) to study the effects produced by the presence of a polyethylene catheter. Phenol red served as a clearance substance. No alterations in renal-clearance values appeared in three animals during or after introduction of the catheter. Urine excretion was markedly reduced in one cat in which the catheter was placed about 1.5 cm in the renal artery. A spasm in the renal artery could be seen upon direct inspection. The same investigators placed catheters in both the renal artery and the renal vein of nine dogs and were able to measure renal blood flow. This value was not affected by the procedure used.

Observations have been made during experimental selective angiography on dogs which suggest that a catheter introduced into the renal artery can occasionally alter circulation through the organ.

In the present work dealing with the study of renal function in dogs by means of selective angiography of the renal artery, values have been obtained for glomerular filtration rate, renal plasma flow, and extraction values for PAH. Inulin and PAH clearance as functional tests were carried out before, during, and after catheterisation of the renal artery.

Methods.

A. *Method of obtaining urine from each kidney separately and marking the renal artery.*

Female dogs were prepared by the technique of McCAUGHAN and MANN (1931) in order to enable repeated clearance tests to be carried out. One ureter with the surrounding bladder wall was transplanted to the abdominal wall while the other ureter opening into the bladder was left intact. The left ovarian vein was also ligated at this time to prevent contamination with blood from this vessel which could affect the extraction values for PAH during subsequent selective catheterisation of the renal vein. In addition, a silver ring was placed about the renal artery at its origin from the aorta to facilitate precise orientation of the catheter tip by fluoroscopy during selective arterial catheterisation.

Urine was collected from the bladder through a Foley catheter. A metal ring, 5 cm in diameter, was sutured to the abdominal skin about the uretral orifice on the prepared side. Urine ran freely into this ring and could be drawn off by a water suction apparatus. A detailed description of the surgical technique and the method of collecting urine as well as clearance values for PAH and inulin can be obtained from a previous paper.

B. Percutaneous selective catheterisation of the renal artery.

Selective renal catheterisation has been carried out in much the same manner described by EDHOLM and SELDINGER (1956) for human beings. Full details concerning the instruments and technique are given in their paper. For the present work polyethylene catheter and a Seldinger needle no. 160 have been used. The distal extremity of the catheter was heated and bent to form a semicircular curve with a radius of about 1 cm. The radius has varied somewhat to suit the size of the dog. The catheter was introduced into the femoral artery by Seldinger's method and the entry guide was replaced by a carefully-measured guide, the extremity of which extended to the tip of the catheter. The polyethylene

Fig. 1. Catheterization of renal veins and renal artery. Radio-opaque catheters in left and right vena renalis and polyethylene catheter with leader, in right renal artery.

catheter prepared in this fashion was radio-opaque. Since the guide was flexible, the bend in the catheter was not disturbed. The animals were placed on their backs for entry into the artery and fluoroscopy. By carefully advancing and withdrawing the catheter tip in the L I to L III region of the aorta during fluoroscopy, it is readily apparent when the catheter reaches the origin of the renal artery. (See Fig. 1.) With practice, insertion of the catheter into the artery requires only a few minutes.

C. Experimental arrangements.

The dogs were fasted for the twenty-four hours preceding an experiment. Five hundred ml water were given by stomach tube 30 min before anaesthesia with mebumal® was induced. A polyethylene catheter no. 160 was placed in a vein of a foreleg and the anaesthetic was administered through this. The animals were placed on their backs and intubated with a Maghill tube. During the entire experiment the dogs were maintained under light anaesthesia. The depth of anaesthesia was checked by means of the corneal and the toe reflexes and additional small doses of mebumal were given as required. The experimental procedure never extended over more than three hours.

Table I.

Results of function tests before, during and after catheterisation of renal artery.

The figures referable to the catheterised kidney during catheterisation itself are italicized.
BP = basal period; CP = catheterisation period; PC = post catheterisation period.

Dog	Expt no.	Periods	Right kidney					Left kidney				
			Urine ml/min	C_{In} ml/min	C_{PAH} ml/min	$\frac{C_{In}}{C_{PAH}}$	E % PAH	Urine ml/min	C_{In} ml/min	C_{PAH} ml/min	$\frac{C_{In}}{C_{PAH}}$	E % PAH
F 13	48	BP	1.3	32	229	0.14	83	1.1	32	217	0.15	75
			0.9	21	122	0.17	81	0.9	22	130	0.17	76
		CP	1.0	50	158	0.31	78	*1.8*	*60*	*199*	*0.30*	*78*
		PC	1.5	75	142	0.53	78	1.8	83	151	0.55	81
			0.9	45	115	0.39	83	1.2	50	133	0.38	86
			1.0	70	131	0.53	89	0.9	55	112	0.49	86
F 13	50	BP	2.0	34	101	0.34	93	1.8	29	84	0.34	93
			2.0	32	110	0.29	95	1.5	24	86	0.28	91
		CP	*2.2*	*42*	*149*	*0.28*	*85*	1.5	36	107	0.34	85
		PC	2.4	45	148	0.30	87	2.5	34	130	0.26	90
			2.0	36	114	0.32	91	2.4	34	108	0.31	91
F 17	43	BP	1.3	46	122	0.38	91	1.3	42	93	0.45	
		CP	1.0	44	66	0.67	88	*1.5*	*54*	*71*	*0.76*	
		PC	0.8	30	62	0.48	84	1.2	37	73	0.50	
F 20	47	BP	1.6	35	124	0.29	95	1.7	49	144	0.34	98
			2.3	34	167	0.20	89	2.4	43	179	0.24	94
		CP	2.7	48	192	0.25	89	*2.7*	*47*	*226*	*0.21*	*89*
		PC	2.2	28	209	0.13	91	2.0	30	239	0.13	83
			1.5	41	162	0.25		1.1	40	179	0.22	
			1.9	46	163	0.28	97	1.5	42	170	0.25	
F 20	49	BP	1.6	35	95	0.36	80	2.1	35	102	0.34	80
			1.8	40				1.8	43			
		CP	1.9	37	106	0.35	83	*2.9*	*46*	*136*	*0.41*	*70*
		PC	2.5	45	121	0.37	83	2.9	44	123	0.36	74
			2.8	35	115	0.30	82	3.1	37	132	0.29	71

Table I (continued).

		CP	1.9	37	106	0.35	83	*2.9*	*46*	*136*	*0.41*	*70*
		PC	2.5	45	121	0.37	83	2.9	44	123	0.36	74
			2.8	35	115	0.30	82	3.1	37	132	0.29	71

Table I (continued).

Dog	Expt no.	Periods	Right kidney					Left kidney				
			Urine ml/min	C_{In} ml/min	C_{PAH} ml/min	$\frac{C_{In}}{C_{PAH}}$	E % PAH	Urine ml/min	C_{In} ml/min	C_{PAH} ml/min	$\frac{C_{In}}{C_{PAH}}$	E % PAH
F 22	52	BP	2.4	33	127	0.26	90	1.2	26	93	0.28	88
			3.1	39	141	0.28	85	1.4	27	107	0.25	83
			5.2	46	167	0.28	78	2.8	30	128	0.23	80
		CP	*0.1*	*0.4*	*1.2*	*0.30*	*43*	1.0	19	90	0.21	
		PC	1.9	25	140	0.18	77	0.9	19	94	0.20	81
			1.5	26	117	0.22	84	0.6	19	84	0.23	76
			1.7	28	97	0.29	88	1.2	20	68	0.29	80
F 22	57	BP	0.8	36	127	0.28	88	0.4	27	90	0.30	72
			0.6	29	92	0.32	86	0.4	27	86	0.31	80
		CP	6.4	38	110	0.35	90	*0*	—	—	—	19
		PC	4.6	34	108	0.32	87	0.8	24	97	0.25	78
			1.7	26	99	0.26	88	0.8	26	111	0.26	77
			1.6	33	117	0.28	87	0.8	27	95	0.28	81
F 22	66	BP	0.5	50	130	0.39		0.3	32	93	0.34	
			1.0	44	97	0.45		0.2	37	92	0.40	
			1.7	40	76	0.53		0.4	37	76	0.49	
		CP	*1.9*	*34*	*94*	*0.36*		0.3	39	73	0.53	
		PC	0.6	29	80	0.36		0.4	35	67	0.52	
			0.4	28	83	0.34		0.3	29	58	0.50	
			0.3	30	73	0.41		0.2	40	96	0.42	
F 25	61	BP	0.4	34	114	0.30		0.3	29	106	0.27	76
		CP	0.4	26	81	0.32		*0.6*	*23*	*96*	*0.24*	*86*
		PC	0.5	21	81	0.26		0.3	14	50	0.28	88
			0.4	26	80	0.33		0.5	33	111	0.30	84
F 32	74	BP	1.0	35	69	0.51		0.5	11	40	0.28	61
			1.4	42	92	0.46	78	0.5	17	65	0.26	64
		CP	1.5	42	89	0.47	82	*0.3*	*1.6*	*5.8*	*0.27*	*50*
		PC	1.2	33	82	0.40	83	0.4	14	60	0.23	58
			0.8	32	94	0.34	82	0.4	12	57	0.21	59

After the induction of anaesthesia, polyethylene catheters no. 160 were placed in each femoral artery. One of these arterial catheters was connected to a kymograph for the recording of blood pressure while the other was utilised only for catheterisation of the renal artery. Vein catheters were then introduced into the renal vein, either unilaterally or bilaterally, in the manner described by HELANDER, ÅSHEIM and ÖDMAN (1958). See Fig. 1. Physiological saline solution was continuously infused through these vein catheters at a rate of 80 drops per minute to prevent clotting. Clearance tests were commenced by giving an initial dose of 10 ml inulin and 1.5 ml PAH corresponding to 40 and 15 mg per kg body weight respectively. Inulin and PAH were then infused continuously through the catheter inserted into one of the leg veins. The plasma concentration of PAH was maintained at about 2 mg per cent and that of inulin, between 15 to 25 mg per cent. Forty minutes after infusion was commenced, clearance periods lasting 15 or 20 min were begun. Blood samples were taken simultaneously from the femoral artery and the renal vein at the midpoint of each period. Urine for each period was collected through a Foley catheter permanently placed in the bladder. The bladder was evacuated manually with constant pressure until no more urine could be expressed. Known amounts of saline were then used to rinse the bladder. As a final measure, 15 to 20 ml air were repeatedly pumped into the bladder and the last drops of urine and saline expelled. Urine from the transplanted ureter was collected in the manner described under surgical technique.

The catheterisation experiments were carried out as follows. After a varying number of control periods (see Table I), the clearance tests were discontinued and the arterial catheter was introduced into the renal artery under fluoroscopy. When it was in position, a new period of 20 min was begun. At the end of this period, the catheter was withdrawn from the vessel and clearance tests were carried out in two or three 20 min periods. The total time during which the catheter was present in the renal artery was about 25 min. The outer diameter of the arterial catheter was 1.6 mm and the inner diameter of the renal artery of the dogs examined varied between 4.5 and 5 mm. This measurement was obtained roentgenologically during subsequent angiography of the animals. The cross-sectional area of the catheter was less than 12 per cent of the cross-sectional area of the vessel.

D. Analytical methods for determination of the clearance agents.

JOSEPHSON and GOLDIN's (1943) modification of CORCORAN and PAGE's (1939) method was used for the inulin analysis. SMITH's (1951) method was used for PAH determinations. All analyses were performed with the aid of a Beckman spectrophotometer, model B.

Experimental series.

Six bitches weighing between 18 and 25 kg were used. The urine of these animals was routinely checked for specific weight, protein, glucose, and sediment. Values for RBC and total WBC, Hb, haematocrit, NPN,

and sedimentation rate were also obtained. Only animals with normal values were obtained.

Catheterisation experiments were repeated at weekly or biweekly intervals on three of the animals in this series. In one of these dogs (F 32), moderate stasis occurred in the transplanted ureter on one occasion (see Table I) as a result of oedema about the uretral orifice following slight trauma during the induction of anaesthesia. The temporary nature of the stasis was apparent from the fact that reinvestigation one week later gave normal results with good agreement between the clearance values for both sides. The functional investigations of these animals covered five to seven clearance periods except in one instance for which values for only three periods were obtained. In most cases both renal veins were catheterised as can be seen from the extraction values in Table I.

Results.

Examination of the values given in Table I shows that a catheter inserted into the renal artery occasionally can give rise to functional disturbances both in the kidney in question and in the contralateral kidney. These disturbances can involve diuresis, GFR, or RPF. Parallel depression or increase of the functional values for both kidneys did not invariably occur. Reduction of diuresis could be quite pronounced (see F 22 (57), F 32 (74), F 22 (52) in Table I. The figures in brackets indicate the experiment number.). The values for filtration and renal blood flow in the latter two experiments were also greatly reduced. Instances were also seen of elevated GF and simultaneously reduced blood flow through both kidneys (F 17 (43)) and of elevated GF and increased RPF (F 13 (48)). Slightly elevated or decreased renal function values were obtained in other instances as well but the deviations were not so pronounced as in the examples cited. F 22 (52), F 22 (37), and F 32 (74) showed reduced extraction values for PAH during oliguria or anuria.

It has been mentioned previously that functional disturbances can also occur in the contralateral kidney. F 17 (43), for example, showed reduced renal blood flow and F 13 (48) both increased glomerular filtration and increased renal blood flow. An obvious depression of function in the contralateral kidney is illustrated by F 22 (52). Compensatory increase of diuresis can also occur (F 22 (57)). In this instance it appears that anuria in the catheterised kidney stimulated a pronounced diuresis in the contralateral kidney.

Abnormality of renal function is not necessarily limited to the time that the catheter is present in the renal artery. The abnormalities may persist throughout the subsequent control periods after withdrawal of the catheter. F 22 (52) demonstrates a continued, pronounced effect upon the contralateral kidney while the catheterised kidney shows a definite tendency to return to the original functional values. An independent increase of glomerular filtration can be observed during the first control period after withdrawal of the catheter from dog F 13 (48). Reduction of both glomerular filtration and renal blood flow was obtained for the catheterised kidney during the first post-catheterisation control period in dog F 25 (61) while the contralateral kidney showed unchanged values.

Discussion and Conclusions.

Clearance studies reflect only indirectly the state of affairs within a kidney through the information which can be gained about renal blood flow, filtration activity, and the capacity for excretion and resorption. Many types of haemodynamic and nervous changes can give similar values during such studies. Reduced glomerular filtration and renal blood flow, for example, can result from a depressed systemic blood pressure. Similar changes for clearance values, however, may also be an expression of vasoconstriction in arterioles, and afferent or efferent vessels. The mere presence of the catheter in the renal artery naturally reduces the available lumen of the vessel to a certain extent. In the case of the animals used for the present experiments, the catheter took up about 12 per cent of the total cross-sectional area of the vessel. LINDELL and OHLIN have examined 9 animals in which the catheter occupied 10 and 20 per cent respectively of the cross-sectional area. They found no differences in renal blood flow when a comparison was made before and during catherisation by direct measurements in the renal vein. Accordingly, it is improbable that the catheter used for the present experiments affected blood flow to the kidneys on account of its size.

Alterations in renal function, *i. e.* reduced diuresis, renal blood flow, glomerular filtration, and extraction values, were observed on three occasions during these catheterisation experiments. Furthermore, an increase in glomerular filtration was observed

in one case and in another experiment, glomerular filtration of the same magnitude as during the control periods occurred concomitantly with reduced renal blood flow. The values obtained are open to several interpretations. A reduced renal blood flow and extraction value may reflect a lowered blood pressure in the kidney produced by a local contraction of the renal artery at the site of the catheter tip. Such contractions have been observed by LINDELL and OHLIN (1957) in cat. LINDBOM (1957) and WICKBOM and BARTLEY (1957) have reported similar changes in peripheral vessels. During investigations into haemorrhagic and traumatic shock in dogs PHILLIPS *et al.* (1946) and SELKURT (1946) found a reduction in renal blood flow and glomerular filtration and low extraction values when blood pressure was depressed to about 70 mm Hg. They postulated that arteriolar constriction occurred during low blood pressure.

In those experiments in which arterial catheterisation affected the clearance values, it is quite possible that the catheter produced reactions other than fall in blood pressure. One possibility, reflex spasm of the efferent vessels could explain the reduced renal blood flow with maintenance of glomerular filtration which was seen in some experiments. Other factors must have been effective in the experiments in which an increase of both renal blood flow and glomerular filtration was obtained.

It has been pointed out previously that clearance tests are only an indirect reflection of the events taking place within a kidney. Consequently, a detailed analysis of all the factors involved is not possible on the basis of the present experiments. It appears likely, however, that reduction in blood pressure, vasoconstriction within the kidneys, or both these factors together may have been initiated by the arterial catheter. The function of both the catheterised and the contralateral kidney can be affected.

The clearance studies show that a kidney could recover its normal function after withdrawal of the catheter from the renal artery. On occasion, the contralateral kidney remained affected during a few clearance periods.

Much of this work was carried out during the time Professor B. Carlström was head of the department of medicine, Veterinärhögskolan. We should like to thank him for the generous support and helpful advice he has given us.

Summary.

Insertion of a catheter into the renal artery occasionally affects renal function by producing pronounced alterations in renal blood flow and glomerular filtration, and the ratio C_{In}/C_{PAH}. On other occasions, no definite effect can be observed. Effects may also be produced in the contralateral kidney. A detailed analysis of the initiating mechanism was not possible with the experimental method described in this paper.

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Slow Membrane Potential Changes Accompanying Excitation and Inhibition in Spinal Moto- and Interneurons in the Cat during Natural Activation.

By

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Introduction.

In 1938 BARRON and MATTHEWS presented the fundamental observations that reflex discharges in a spinal ventral root are preceded by a slow electrotonic potential and that the frequency of the motor nerve discharge is related to the rate of rise and the level of this potential. They concluded that a given motoneuron fires repetitively in response to a steady depolarization built up by activity in terminals of afferents or interneurons. Stimuli causing inhibition of impulse discharges were found to result in a fall of the potential level produced by the excitatory stimulus, but inhibitory stimuli alone were never observed to cause a positive potential shift.

This indirect method of measuring slow membrane potential changes of spinal motoneurons by recording their electrotonic spread in the ventral roots has since been utilized by several investigators[1]. In experiments on the cat's spinal cord BERNHARD (1947) showed that a synchronized afferent volley may result in a

[1] For references see various papers on spinal cord in Supplement 106 of Acta Physiologica Scandinavica 1953, "In Honour of S. Ramón y Cajal".

slow polyphasic potential composed of both negative and positive potential fractions, and reflex testing revealed that the motoneuron excitability is influenced in an opposite way during the two potential phases. The potentials were compared with certain changes in the L-fraction of the nerve membrane potential (cf. LORENTE DE NÓ 1947).

Similar slow ventral root potentials, not dependent on any previous impulse discharges, can also be produced by suprasegmental activation, especially when repetitive stimulation is applied (BERNHARD and SKOGLUND 1947). It is then possible to obtain ventral root potentials of considerable amplitude which are either negative or positive in relation to the initial level, and typical sequences of rhythmical oscillations may also be set up. Long-lasting shifts of the ventral root potential level in either direction can also be obtained by natural changes of the proprioceptive inflow, *e. g.* simply by altering the position of one of the hindlimbs of the experimental animal, and such shifts were found to be associated with typical changes in the excitability of the spinal motor systems (BERNHARD, SKOGLUND and THERMAN 1947).

BARRON and MATTHEWS (1938) also showed that a constant current applied in such a way as to produce depolarization of the central part of the motoneuron caused repetitive firing of the neuron, indicating that "there is some structure of the motoneuron which readily responds rhythmically to a direct current". Further evidence in favour of such a mechanism of impulse generation was obtained by the demonstration of inherent autorhythmical properties also in the peripheral part of the cat's motoneuron (SKOGLUND 1942).

In spite of several investigations along these lines demonstrating similarities between rhythmical discharges induced by artificial depolarization and natural activation of spinal motoneurons (*e. g.* KUGELBERG and SKOGLUND 1946, ALANIS 1953, HYDE, KUGELBERG and SKOGLUND 1953, ALVORD and FUORTES 1953) opinions have still differed as to the role of steady depolarization in the rhythmical firing. From studies of monosynaptic reflexes elicited by single afferent volleys it has thus, *e. g.*, been concluded that long-lasting events only exert facilitation and that firing of the neuron requires a specific short-lasting action (LLOYD 1946, cf. also 1955). In recent investigations the origin of motoneuron rhythms has been taken up for further analysis and discussion (FUORTES 1954, MORRELL *et al.* 1956, FUORTES 1957). The results ob-

tained support the view that rhythmical reflex firing is a result of a steady action which is transformed in the motoneuron into oscillatory events.

The intracellular recording technique, successfully applied by ECCLES and collaborators in profound analyses of single impulse reflexes (see ECCLES 1953, 1957), may evidently be expected to provide further information of the mechanisms of the initiation of rhythmical impulses. The present paper deals with such studies on moto- and interneurons in non-anesthetized spinal cat preparations in which sustained activity was induced by application of various types of natural stimulation (cf. KOLMODIN and SKOGLUND 1954, KOLMODIN 1957).

The first section gives a description of the slow potential changes during natural activation of spinal neurons, of fundamentally the same type in moto- and interneurons, and their relation to the spike potentials. Special attention is paid to the post-spike repolarization phases.

The second section gives an account of the slow membrane potential changes associated with inhibition of previously set up discharges during natural and artificial activation of inhibitory sources.

The third section presents an analysis of the miniature potentials and their variations with changes of the afferent inflow and of the membrane potential level.

Methods.

Decapitate, slightly curarized cats kept on artificial ventilation have been used. In all experiments the ipsilateral ventral roots from L_4 to S_1 were arranged for antidromic stimulation necessary to identify motoneurons. In some experiments the hindlimbs were kept intact except for fixation at hip or knee, in others the tendons to certain muscles were dissected free so that stretch could be applied. In a few experiments the sural or gastrocnemius nerve was prepared and put on stimulating electrodes.

As the recording technique has been described in detail by HAAPANEN, KOLMODIN and SKOGLUND (1958), only the following principal data are given in this connection: capillary electrodes filled with 2.7—3.0 M KCl solution were connected to an input stage of cathode follower type (HAAPANEN and OTTOSON 1954). The main amplifier consisted of a direct coupled amplifier deflect-

ing one beam of a double beam oscilloscope as well as an inkwriter (Siemens, type SD 12 K, response time 1/sec) for continuous recording of the resting potential. In order to permit studies of action potentials at higher amplification an a. c. coupled amplifier, deflecting the other beam of the oscilloscope, was also connected to the output of the main amplifier. In a few experiments a counting rate meter in combination with an inkwriter of the same type as above was used for a recording of the discharge frequency (cf. KOLMODIN 1957).

Note on nomenclature.

Throughout this paper all membrane potential values have been calculated from the zero line at the start of the experiment; the sources of error thus involved have been discussed in a previous paper (HAAPANEN, KOLMODIN and SKOGLUND 1958).

All level indications refer to the membrane potential value; thus, *e. g.*, a membrane potential level corresponding to 60 mV is always referred to as being higher than one of 50 mV although in the records seen below the latter.

As the "true" resting value of a membrane potential is difficult to determine (cf. Discussion, Section I) we have preferred to use the term repolarization for all processes involving an increase of the membrane potential value.

Results.

I. Membrane potential changes during activation.

1. Changes in membrane potential level during sustained rhythmical activity as revealed by inkwriter recordings.

The method of recording the membrane potential level continuously by means of an inkwriter, used as a routine technique in our experiments, is well suited to give a general survey of the spontaneous variations as well as of the changes in membrane potential level induced during sustained natural activation.

Fig. 1 shows sections of such a recording from a motoneuron during a 15-minute period. In the absence of peripheral stimulation variations occur within a rather limited range, the lowest membrane potential value recorded being 58, the highest 61 mV (upper record). Such variations may partly be due to changing

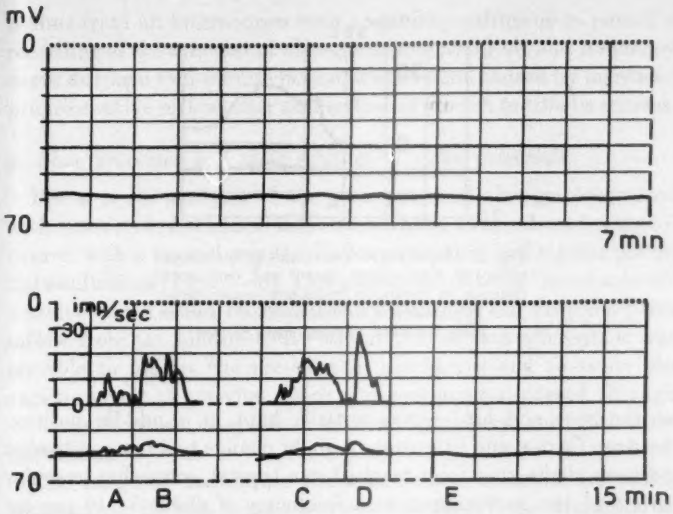

Fig. 1. *Motoneuron.* Spontaneous and induced variations in membrane potential level. *Upper record* spontaneous variations; *lower record* depolarization caused by change of afferent inflow from ipsi- or contralateral hindlimbs: at *A* slight extension of contralateral hindlimb at knee-joint; at *B* full extension of same limb; at *C* activation from ipsilateral side; at *D* from contralateral hindlimb. *E*, control record showing small range of variations after cessation of stimulation. *Inset* diagrams show frequency of spike discharge. *Time* in minutes represents total period of continuous recording, irrelevant parts of which have been cut out at gaps in lower record. *Time* between vertical lines 30 sec.

recording conditions (cf. HAAPANEN, KOLMODIN and SKOGLUND 1958), but slowly alternating changes like those shown in this record may also be attributed to physiological variations in the membrane potential level occurring in non-anesthetized preparations in which the motoneurons are subjected to varying impinging activity. Whatever factors may contribute to these potential changes, the motoneuron did, however, not discharge impulses within this range of variations.

This initially silent cell could be brought to fire by changing the afferent inflow from the hindlimbs — simply by passive limb movements — and the sustained discharges thus induced were accompanied by changes in the membrane potential level which stand out clearly (lower record) as compared with the spontaneous variations. The contralateral hindlimb — intact except for fixation

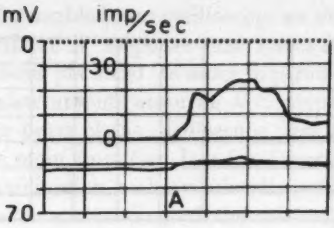

Fig. 2. *Interneuron.* Variations in membrane potential level (*lower curve*) and concurrent changes in discharge frequency (*upper curve*). At *A* application of stretch to tibialis anticus. *Time* between vertical lines 30 sec.

at the knee and hip — was initially kept in a middle position between flexion and extension. A slight change to a more extended position at the knee-joint resulted in a typical, somewhat irregular firing of the motoneuron at a frequency of about 5—10 per sec which was maintained as long as the limb was kept in this new position (see inset of frequency diagram at *A*). Full extension of the limb caused a higher discharge frequency of up to 20 per sec (*B*). It is evident that during the same period definite changes occur in the membrane potential level parallel with the frequency changes, although the inkwriter recordings do not allow of any accurate quantitative comparisons (cf. Methods).

The concomitant changes in the frequency curve and polarization level at *C* are produced by a similar activation from the ipsilateral side, while the change at *D* is again produced by a passive movement of the contralateral hindlimb. The last part of the figure represents a control record of some minutes' length, showing that after the stimulation has ceased the membrane potential variations are once more limited to the same small range as before the activation set in.

Since the membrane potentials recorded from interneurons are, as a rule, of lower value and of less stability (HAAPANEN, KOLMODIN and SKOGLUND 1958), small potential changes induced in these neurons by natural stimulation are more difficult to follow during periods of some length. However, from a series of observations made it can be concluded that fundamentally the same slow potential changes as can be demonstrated in motoneurons may also accompany naturally induced activity in interneurons. Fig. 2

is thus from an interneuron with a stability sufficient to permit a recording of the concurrent changes in potential level and frequency curve during a two-minute period of activation caused by increased proprioceptive inflow after application of stretch to tibialis anticus.

2. Slow processes and their relation to spike potentials.

For a closer analysis of the slow potential changes associated with sustained rhythmical activity we have first selected a motoneuron with a typical regular discharge rhythm and regular potential oscillations (Figs. 3—8). This neuron exhibited a "spontaneous" activity which could be increased by changing the proprioceptive inflow from the hindlimb. By stimulation of skin afferents it was possible to inhibit the pre-existent discharges and to study the various levels of activity which this motoneuron passed through as a result of an interaction between inhibitory and excitatory influences.

The range of membrane potential values recorded from this motoneuron in non-firing state was 65—52 mV, the average level for the initiation of rhythmical activity being 51 mV. The records in Fig. 3 illustrate some very slow membrane potential changes — from subthreshold level up to firing level — as a result of a gradually increasing excitation. In record *A* the membrane potential decreases at a rate of about 5 mV per sec from the initial value of 58 mV to 51 mV, at which level a rhythmical firing of about 10 per sec is initiated. Record *B* shows the same slow depolarization process but in this case an occasional spike is fired even at 53 mV. Before this first spike has been fired an increase in the small baseline oscillations can be observed (cf. Section III), and during the interval between this spike and the regular firing at 51 mV a tendency is also apparent towards larger fluctuations (arrows) which seem to be of about the same duration as the spike interval in the later repetitive firing.

The falling spike phase continues beyond the firing level into a repolarization phase reaching 65 mV. From this bottom value the membrane potential is then brought back at a constant rate to the firing level. This latter phase which represents a "re-depolarization" (cf. ARVANITAKI and CHALAZONITIS 1956) will in the following be referred to as the *depolarization phase* of the slow inter-spike oscillations.

As illustrated in Fig. 3 the first phase of repolarization beyond the pre-spike membrane potential level appears to be as rapid as

Fig. 3. *Motoneuron.* Slow membrane potential changes during increasing excitation. *A*, slow depolarization from initial level of 58 mV to rhythmical firing level of 51 mV. *B*, same slow depolarization but with occasional spike firing at 53 mV. See text. Upper parts of spikes cut off. *Dotted line* at 65 mV. *Time bar* 100 msec.

Fig. 4. Same motoneuron as in Fig. 3. Successive changes in firing and repolarization levels during increasing activity, the discharge frequency rising from 10 to 20/sec. *Dotted lines* at 50 and 65 mV. *Time bar* 100 msec.

the preceding falling phase of the spike and there is, as a rule, a marked transition to a following slow repolarization phase. Thus, for instance, it is quite clear that the quick repolarization of the first spike in Fig. 3 *A* ends at 60 mV (arrow). In the following the corresponding membrane potential level will be referred to as the *quick repolarization level*. The total rapid potential change from the spike peak to this level represents the *quick repolarization phase*, which for the present may be considered as a more or less uniform process. The repolarization maximum (in this case 65 mV) will be termed the *slow repolarization level;* the *slow repolarization phase* corresponds to the small potential change between the two levels (cf. diagram in Fig. 5).

The division into a quick and a slow repolarization phase may be more or less marked in records from different neurons. Other observations suggest, however, that a differentiation between two phases may be justified; thus, for instance, a slow repolarization may sometimes appear alone (cf. Fig. 8).

Fig. 4 shows a recording during a period of successively increasing activity of the motoneuron, in which the discharge frequency rose from about 10 to 20 per sec. The continuous lowering of the slow repolarization level, from 63 to 56 mV, is obvious. There is also a lowering of the firing level with the increase in frequency, although this diminution is smaller than the change in repolarization level.

The four records *A—D* in Fig. 5, selected from another activation phase of the same motoneuron, represent a wider range of frequency variations. Corresponding to a rise in frequency per sec of 17.5 there is a change in firing level of about 3 mV, while the repolarization level is lowered 8 mV. In the diagram below the records tracings of the average membrane potential oscillations

Upper parts of spikes cut off. *Dotted line* at 65 mV. *Time bar* 100 msec.

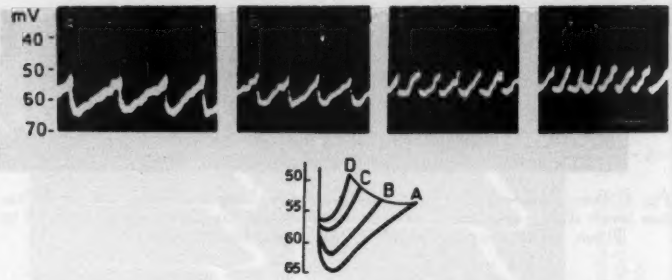

Fig. 5. Another activation phase of same motoneuron, illustrating wider range of frequency variations (*A*—*D*, as per below). The values given are average values and — like those plotted in Fig. 6 — obtained by measurements on projections of the recording film on mm paper at a magnification corresponding to 1 mV per mm. The firing level has been measured at the point where the spike takes off from the upper edge, and the repolarization phase from the lower edge, since these measures can be determined with the greatest accuracy. The difference between these two levels will thus comprise the baseline width and small oscillations. *Diagram below records:* superimposed tracings of average membrane potential oscillations (upper baseline contour) in *A*—*D*. *Time bar* 100 msec. See text.

		A	*B*	*C*	*D*
Slow repolarization level	mV	65	62	58.5	57
Quick repolarization level	»	59	58	57.5	56
Firing level	»	52	51	49.5	49
Frequency per sec		8.5	13	19.5	26

in *A*—*D* are superimposed, allowing a direct comparison of the relative changes of the various phases. As appears from this diagram, the change in the level of the quick repolarization is relatively small (from 59 to 56 mV) as compared with that of the slow repolarization (from 65 to 57 mV), and the result is that at higher frequencies (*D*) the slow phase only just exceeds the level of the quick phase.

In Fig. 6 are plotted variations in firing and slow repolarization levels during four subsequent periods of activation of increasing intensity (*I* — *IV*). As seen in the diagram, there was a remarkable constancy in the initial discharge frequency (10 per sec) and in the slow repolarization level (which at the beginning of each period was 65 mV), whereas the initial firing level showed small variations (between 51 and 52 mV). During the first activation period there were insignificant, irregular changes in frequency and firing level but possibly a tendency towards a decreased value of the slow repolarization level. In the second activation period there was

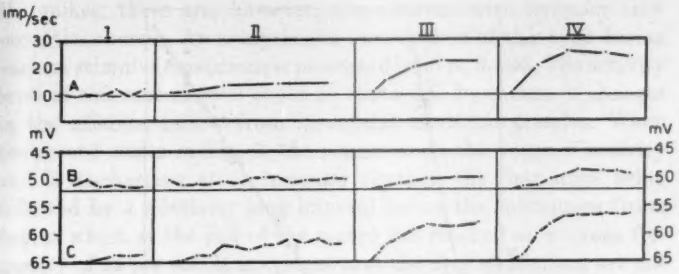

Fig. 6. Diagram showing changes in discharge frequency (A), firing (B) and slow repolarization (C) levels of motoneuron during four subsequent periods of activation of increasing intensity (*I*—*IV*). Each value in the frequency curve is based on average of five successive spike intervals. Full explanation in text.

a definite change in frequency from 10 to 14 per sec; this is clearly reflected in the repolarization level, and also the average firing level is changed. With the more pronounced increase in frequency during periods *III* and *IV* the changes in firing level are also quite evident.

To the right in Fig. 7 (filled circles) the various values of the slow repolarization level in the different activation periods in Fig. 6 have been plotted against the impulse interval (1/frequency), and it appears that within the frequency range studied in this motoneuron the relation is approximately linear. To the left in the figure (open circles) the firing level values have been plotted. As will be seen, there may be great variations in interval at one and the same firing level; thus for instance at 51 mV the intervals varied between 67 and 104 msec. However, the average relation between impulse interval and firing level seems to be about linear. The changes in firing level were in this case relatively small in comparison with the changes in repolarization level. In other motoneurons the firing level may vary to practically the same extent as the repolarization level, as will be illustrated in a following example.

At various levels of sustained rhythmical activity the depolarization phase may occasionally be seen not to attain the average firing level, as illustrated in Fig. 8. When the "deficit" is small, as in record *A*, the result may often be a spike of reduced amplitude, varying from three-fourths to one-tenth of normal. When the "deficit" in depolarization is greater, as in record *B*, no spike at

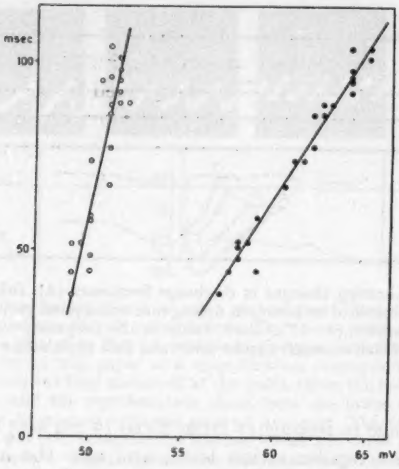

Fig. 7. Collected data from the four activity periods of Fig. 6. Firing levels (*open circles*) and slow repolarization levels (*filled circles*) plotted against impulse intervals (ordinates).

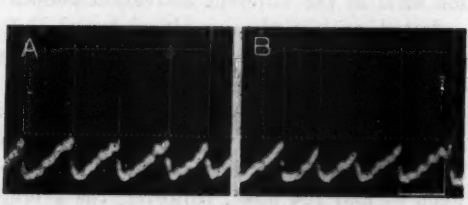

Fig. 8. *Same motoneuron* as in previous pictures. Atypical slow oscillations in sequences of rhythmical discharges. *A*, small "deficit" in depolarization resulting in spike of reduced amplitude; *B*, greater "deficit", no spike visible; *Vertical bar* 10 mV. *Time bar* 100 msec.

all can be seen. It is of great interest that although the firing level is not reached a normal slow repolarization phase follows and that thus the prevailing rhythm of slow oscillations is not disturbed.

The motoneuron described above was characterized by a smooth and regular shape of the potential sequences developing between

the spikes; there are, however, also neurons with *irregular* slow potential courses. An analysis of a motoneuron of this type during various stimulus conditions is presented in Figs. 9—13. The activity level of this motoneuron could be controlled by means of changes in the afferent inflow from ipsilateral hindlimb muscles. When the record starts in Fig. 9, the neuron is on the verge of activity and is discharging at an irregular rhythm, the first spike being followed by a relatively long interval before the continuous firing begins which at the end of the record has reached an average frequency of 12 per sec. It is evident that the slow oscillations are not uniform but vary from one impulse interval to another; especially in the slow repolarization phases of the first 6—7 intervals the variations are quite obvious.

In spite of the irregularity of the slow potential variations a small but definite change both in slow repolarization and firing levels can be seen to occur in Fig. 9 parallel with the increase in activity; when the neuron has reached a higher discharge frequency, as in Fig. 10 *C*, the changes in these levels are quite obvious. It can be shown that there is fundamentally the same relationship between impulse interval, on one hand, and average firing and repolarization levels, on the other hand, as has been illustrated in the previous diagram Fig. 7. However, the range of firing level variations is greater in this motoneuron than in that previously analysed, *viz.* from 56 mV at the lowest activity level in Fig. 9 to an average value of 47 mV at the highest activity level in Fig. 10 *C*.

It is further characteristic of this neuron that the firing level may occasionally show considerable fluctuations at a given activity level, as appears from Fig. 10 *A*, in which variations between 55 and 48 mV can be seen although the changes in frequency are relatively small.

Fig. 10 *B* has been selected to show other variations in the slow oscillations. In this record the slow repolarization phase following upon the first two spikes is missing after the third spike (first arrow), and the result is a continued depolarization nearly to the 45 mV level. It is interesting to see how, in spite of the variations in the slow potential configurations, the spike takes off after the same interval as previously and that only after the next (fourth) spike there is a change of the initial rhythm. At the second arrow this linearly rising depolarization is repeated again, between about the same levels as before, but now at a slower rate of rise, and this time the spike does not take off until a depolarization level of 48

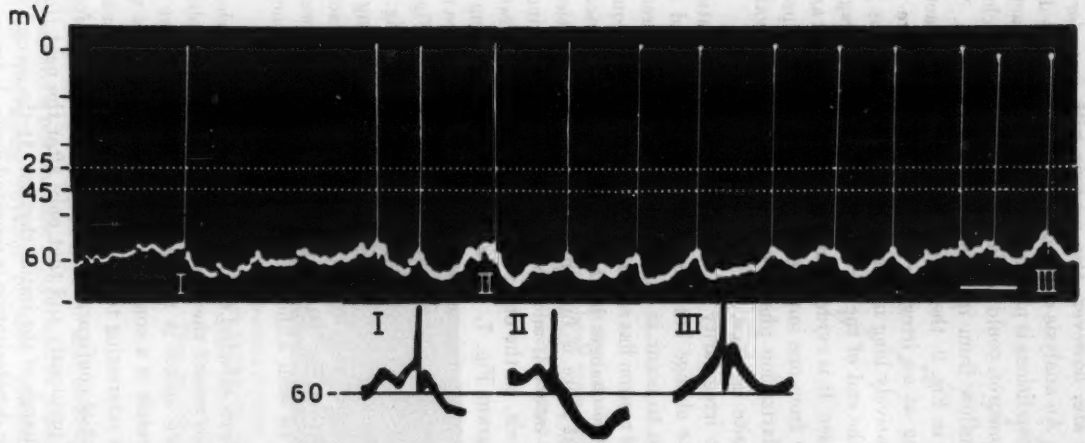

Fig. 9. *Motoneuron*, characterized by irregular slow oscillations. Parallel with increase in frequency to 12/sec there is a change in firing level from 56 (first spikes) to 54 mV (last spikes to right) and a change in maximum slow repolarization level from 65 to 61 mV, while the quick repolarization level remains constant at 60 mV throughout record. Parts of spikes cut out between *dotted lines* (25—45 mV). Diagrams below record show potential sequences at I, II and III schematically drawn on a larger scale. *Time bar* 100 msec. Full explanation in text.

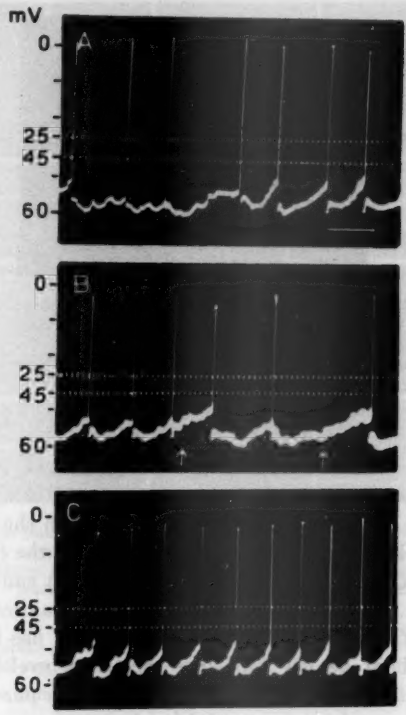

Fig. 10. Same motoneuron as in Fig. 9. *A*, large variations in firing level (between 55 and 48 mV, see text); *B*, atypical slow re- and depolarization phases (see text); *C*, maximum activity level of 18/sec. Average firing level 47 mV, quick and slow repolarization level 58 mV. Parts of spikes cut out in records between *dotted lines* (25—45 mV). *Time bar* 100 msec.

mV has been reached so that an interval of exceptional length results.

Typical of this motoneuron is also that the final phase of the quick repolarization may be more distinctly separated from the slow repolarization phase than in the previous example. The quick repolarization level is comparatively constant during minor variations in the membrane potential level. Thus it remains at about 60 mV throughout the record of Fig. 9 and the result is that when

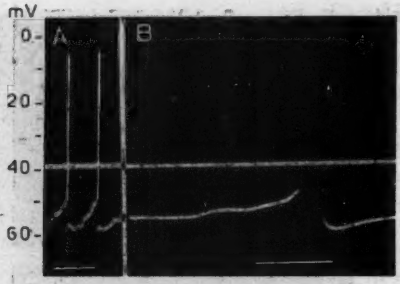

Fig. 11. Same motoneuron as in previous figures. *A*, brief section of continuous recording; *B*, sweep picture of spike from same recording. *Time bar* in *A* 100 msec, in *B* 5 msec. (Upper cathode ray beam at 40 mV.)

the firing level is of a similar high value, as at the beginning of this record, the quick phase, visible on the record below the firing level, is small (at *I*, cf. diagram) or concealed in the first part of the slow repolarization (at *II*). However, when the firing level is lower, as for the last spike in the same record, it can be observed as a separate downward phase (*III*). During maximum activity, as in Fig. 10 *C*, the level of quick repolarization has been slightly lowered to about 58 mV but since the firing level is now at 52 mV the result is a marked quick repolarization phase exceeding the pre-spike membrane potential level.

The spike configuration can be studied on a faster time scale in the sweep record Fig. 11 *B*, which is obtained during a high-frequency discharge of the cell (cf. *A*). The beginning of record *B* illustrates the last part of the preceding depolarization phase with its typical fluctuations. At 50 mV a first steep inflection occurs which at 47 mV changes into the fast ascending spike phase. The level of the spike peak is 10 mV below zero (cf. below). It is evident that the descending phase of the spike continues, beyond the take-off level of the spike, to a quick repolarization level of 59 mV.

A comparison with spikes evoked by artificial ortho- and antidromic stimulation is obviously of interest. In Fig. 12, records *A* and *B* show orthodromic spikes elicited at varying membrane potential levels within the range of the natural firing level variations; except for the brief postsynaptic potential, characteristic of arti-

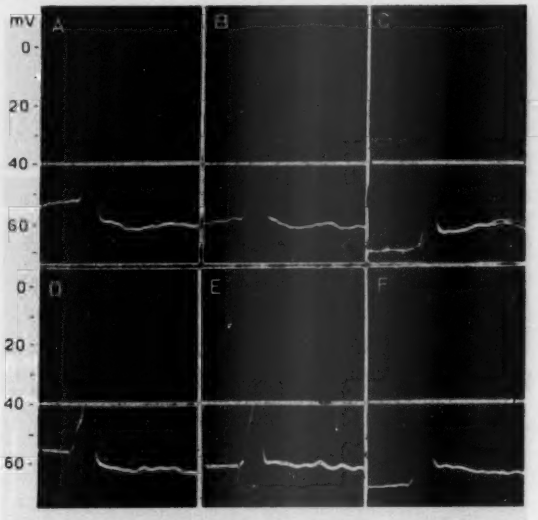

Fig. 12. Same motoneuron as in previous figures. Spikes elicited by artificial orthodromic (*A*—*C*) and antidromic (*D*—*F*) stimulation at varying levels of natural depolarization, illustrating variations in quick repolarization phase.

	A	*B*	*C*	*D*	*E*	*F*
Pre-spike potential level . . mV	53	58	70	54	60	68
Quick repolarization level . »	60	58	63	60	60	62

Time in msec on upper beam at 40 mV.

ficial stimulation (Brock, Coombs and Eccles 1952), the configurations are identical with those of naturally induced potentials. Thus the fast repolarization level shows the same variations as during natural activity, *i. e.* between 58 and 60 mV, which results in a repolarization beyond the pre-spike level in *A* but not in *B* (just as is the case during natural activation, cf. Fig. 9 I, II). With artificial stimulation a spike may be elicited at a higher membrane potential value (62 mV) than during natural activation (58 mV), as shown by record *C*. Although in this case the quick repolarization level has been changed a few mV, to 63 mV, it is anyhow lower than the initial membrane potential level of 68 mV.

A corresponding series of recordings of antidromically elicited spikes (lower records, *D*—*F*) exhibits the same variations in the quick repolarization level as have been found with orthodromic

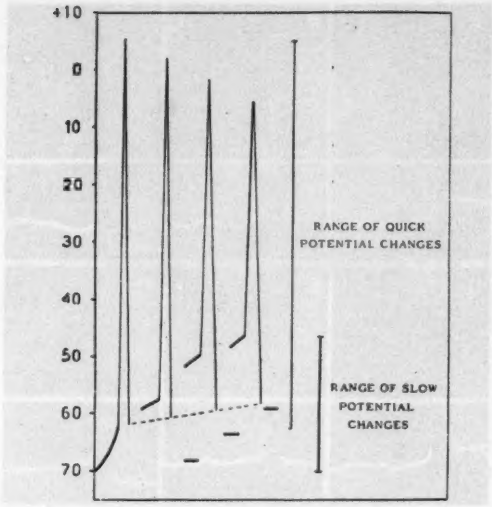

Fig. 13. Diagrammatic representation of quick and slow potential changes of motoneuron, based on records in previous figures. See text. *Dotted line* at quick repolarization levels; slow repolarization levels indicated by *short horizontal lines*.

stimulation; the records have been selected as on the whole matching the upper series in regard to pre-spike membrane potential levels.

From Fig. 12 it appears that the level of the spike peak varies with the pre-spike membrane potential value in a way resulting in considerable changes in the total spike amplitude. Similar variations in spike height occur during natural discharges, as appears from Figs. 9 and 10, and as is further illustrated in Fig. 13. The first spike in this diagram, initiated by an orthodromic volley at the highest membrane potential value recorded, 70 mV, has a total amplitude of 77 mV. The following three spikes, initiated by natural firing at successively lower levels, show a progressive change in peak level, the ascending phase of the last spike measuring only 42 mV.

In the diagram the maximal range of the quick membrane potential changes has been marked off; the limits of variation are from 63 mV below to 5 mV above zero line. The diagram also

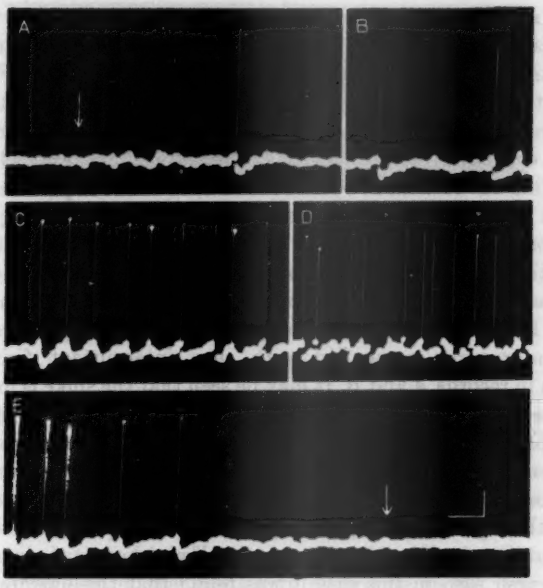

Fig. 14. Same interneuron as in Fig. 2. (Condenser-coupled amplifier.) Sections of continuous recording of sustained discharges elicited by pad pressure. *A* and *B*, slight pressure (applied at arrow) results in small baseline oscillations preceding the first spike. *C* and *D*, discharge elicited by firmer pressure; spike size and repolarization phase can be seen to diminish successively with increasing frequency. *E*, cessation of pressure (at arrow). *Vertical bar* 10 mV. *Time bar* 100 msec.

includes the range of the slow potential changes observed in the same experiment, *viz.* between 47 mV (lowest numerical value of the slow depolarization) and 70 mV (maximal value of the slow repolarization). As may be seen, there is a partial overlapping of the two ranges of fast and slow potential variations.

Although the diagram has been made up on the basis of data from this particular cell with its more irregular slow inter-spike oscillations, it is fundamentally valid also for the type of motoneuron with regular oscillations previously described.

Corresponding analyses of interneurons — so far performed only on a small material — have given essentially the same results as those of motoneurons. In a previous investigation (Haapanen, Kolmodin and Skoglund 1958) it has thus been shown how the

initial repolarization phase following on spikes elicited by artificial stimulation varies with naturally induced changes in the membrane potential level in a way resulting in similar pictures as in Fig. 12.

That the repolarization phases of naturally activated interneurons may be of similar shape and may undergo similar changes as those occurring in motoneurons is illustrated in Fig. 14. This recording has been made by means of a condenser-coupled amplifier with a sufficiently long time constant to permit a recording of the slow oscillations between the spikes. Changes in the membrane potential level were thus not registered, but the records show the changes in amplitude of the repolarization phases in relation to the firing level. To a slight exteroceptive stimulation (pad pressure) this interneuron responded with slow intermittent firing, as shown in records *A* and *B*. It may be seen that the spike is followed by a repolarization phase beyond the baseline which maintains its amplitude as long as the interneuron is discharging at low frequency. Records *C* and *D* show the initial and later periods of the discharge caused by a firmer pad pressure; it appears clearly from these pictures how the amplitude of the repolarization phase beyond the baseline is successively diminished with increasing frequency. Also in this case it was found that the lowering of the amplitude is brought about mainly by a reduction of the slower phase, although in the records reproduced the difference between the two phases is not quite distinct. When the pressure ceases (*E*, at arrow) the frequency is reduced and the repolarization phase increases once more. That there is a reduction of the spike amplitude with increasing frequency is clearly apparent from this series of pictures (cf. especially records *C* and *D*).

Quantitative studies of changes in the firing level during natural activation are rather difficult to perform in interneurons owing to the lower degree of stability of the recorded membrane potential level. However, from some experiments which permitted such measurements it is evident that with increasing frequency the firing level may be raised in the same way as in motoneurons; in one case an increase in frequency from 10 to 80 per sec was observed to correspond to a change in firing level of 2.5 mV.

Fig. 8 above illustrated how occasionally, at various levels of sustained activity in motoneurons, the depolarization did not attain the firing level which resulted in spikes of subnormal amplitude or in a complete drop-out of spikes. The same observation has been made in interneurons, as illustrated in Fig. 15.

Fig. 15. *Interneuron.* Atypical slow oscillations in sequences of rhythmical discharges (condenser-coupled amplifier), resulting in spike of subnormal amplitude (*A*) or no recordable spike (*B*). *Vertical bar* 10 mV. *Time bar* 100 msec.

Discussion.

General comments. Due to the uncertainty prevailing as to the origin and nature of the intracellularly recorded potential components, the interpretation of part of the results must necessarily be tentative. It should be kept in mind that when the electrode is placed in the soma, activity from various parts of the cell is likely to interfere so that not only soma and dendrites but possibly also the proximal part of the axons may contribute to the potentials recorded.

A question of special interest in this connection is the site of initiation of impulses. The earlier concept of a motoneuron impulse set up by orthodromic stimulation as being initiated in the soma (Brock, Coombs and Eccles 1952) had to be modified after a series of investigations indicating that, independent of the mode of activation, a spike potential is primarily set up in the initial segment of the axon from which, secondarily, the soma is invaded (Araki, Otani and Furukawa 1953, Araki and Otani 1955, cf. however Fatt 1957). Various interpretations have been put forward as to the closer mechanism of this secondary invasion (Fuortes, Frank and Becker 1957, Coombs, Curtis and Eccles 1957), but for the purpose of the present discussion this question is less relevant.

A more important consequence of a primary impulse generation in the initial segment is, obviously, that no direct information of the depolarization processes at the actual site of impulse initiation can be obtained from recordings in which the electrode is placed in the soma. However, a close relationship may be presumed to exist between these depolarization processes and the slow mem-

brane potential changes recorded across the soma membrane. Especially the studies of the firing level — which in this investigation has been measured as the membrane potential value at which the initial phase of the impulse recorded in the soma takes off — must be seen against the background of the facts indicated above.

It should finally be pointed out that in an analysis of potentials recorded from the soma one has to consider not only spatially different potential components originating from specific parts of the cell but possibly also various membrane potential fractions which might be attributable to different electrogenetic submicroscopical structures (cf. LORENTE DE NÓ 1947, ARVANITAKI and CHALAZONITIS 1956).

Mechanism of rhythmical firing. There is a great deal of evidence in support of the concept that the rhythmical firing is initiated by the membrane being at a critical level of depolarization brought into a state of slow oscillations, the frequency of which is determined by the inherent properties of the membrane. In the peripheral part of the motoneuron in the cat a rhythmical activity can readily be induced by artificial depolarization, the initial frequency and the type of discharge being very similar to those observed during natural activation (SKOGLUND 1942, HYDE, KUGELBERG and SKOGLUND 1953). In studies of mammalian motor fibres no recordings have so far been made from the site of impulse initiation, but such experiments on other types of nerve with a similar tendency to rhythmical firing have demonstrated the occurrence of local membrane oscillations which primarily determine the discharge rhythm (*e. g.* ARVANITAKI 1939). A similar oscillation tendency of the myelinated nerve membrane has been established by LORENTE DE NÓ (1947) in his studies of electrotonic potentials in frog nerve, and recently also by STÄMPFLI (1956) in recordings from single nodes.

In their investigations on the cat's spinal cord BERNHARD and SKOGLUND (1947) showed that there may occur rhythmical subthreshold oscillations of electrotonic ventral root potentials which had a frequency of 10—12 per sec. In view of this observation it is interesting that subthreshold oscillations have also been found in the intracellular recordings from motoneurons, such as seen in Fig. 3, which are of the same frequency, about 10 per sec, as the subsequent regular spike discharges; these findings may be considered as a further support of the concept that the discharge

rhythm of the motoneurons is preformed by primary slow oscillations of the membrane potential. Oscillations of a similar kind have also been observed in interneurons. Their connection with simultaneously occurring miniature potentials in the two types of nerve cells will be discussed in Section III. As shown in Figs. 8 and 15 above, inter-spike oscillations may occur both in moto- and interneurons of the cat without any spike being recorded, and this fact also speaks in favour of a primary oscillation tendency of slow potential fractions in the membrane.

In this connection we may recall that the existence of a rhythmical prepotential mechanism in other types of nerve cells (*Aplysia*) has been demonstrated by ARVANITAKI and CHALAZONITIS (1955) and TAUC (1955). If there are thus strong reasons to assume that the discharge rhythm in spinal neurons, like in many other nervous substrates, is in the first line determined by a slow oscillation tendency of the membrane, this does not exclude that other processes, such as post-spike excitability changes, may also be involved (cf. ECCLES and HOFF 1932, ECCLES 1953, 1957, FUORTES and FRANK 1955).

Firing level and discharge frequency. For spinal neurons during sustained activity it has previously been generally accepted that the firing level is a fixed level, independent of the state of activity of the cell, and hypotheses as to the mechanism of the rhythmical discharge in motoneurons (ECCLES 1953) have been based on this assumption. However, the results described above show that, with increasing frequency, there is a successive lowering of the critical membrane potential level for spike initiation. It is of interest that this also applies to the slowly adapting type of sensory nerve cell of the crayfish, as has been shown by EYZAGUIRRE and KUFFLER (1955). The example illustrated above in our diagram Fig. 7 indicates that there is a linear relationship between the change in firing level and the impulse interval, implying a hyperbolic relation to the frequency, but before it can be decided whether this relation is generally valid a large number of cells have to be tested. For the impulse initiation in the muscle spindle a linear relationship between depolarization level and frequency has been established (KATZ 1950), but for the present we abstain from a closer discussion of the various reasons for this difference.

Quick and slow membrane potential components. Studies of electrical processes of different time constant in peripheral nerve have led to a

concept of the membrane potential as being composed of several fractions out of which in the first place two main types, one quick and one slow, the Q- and the L-fractions, have to be considered (LORENTE DE NÓ 1947). The spike potential and certain quick electrotonic processes may be regarded as changes in the Q-fraction, whereas afterpotentials and slow electrotonic processes represent fluctuations in the L-fraction. One of the basic results of the analysis is the finding that the excitability properties of the nerve membrane are determined by the relation between the two fractions within the range of the total membrane potential value. Thus, *e. g.*, with a diminishing ratio L/Q the activity of the nerve is increased and a tendency to spontaneous repetitive firing appears.

After having established the membrane properties of the soma and axons of sympathetic B ganglion cells in toad, LAPORTE and LORENTE DE NÓ (1950) performed an analysis of synaptic processes in terms of changes in the two fractions. It appeared that presynaptic impulses may exert a catelectrotonic (depolarizing) as well as an anelectrotonic (hyperpolarizing) action on the sympathetic ganglion cells, and these postsynaptic potentials were found chiefly to represent changes in the L-fraction of the soma.

We are of course well aware that data on the membrane properties of the frog nerve and sympathetic ganglion cells of toad cannot be directly applied to mammalian spinal neurons; however, in the analysis of the quick and slow potential changes, as diagrammatically represented in Fig. 13, it has proved to be a useful working hypothesis to think of them as being variations in a quick and a slow membrane potential fraction; to what extent these postulated fractions actually correspond to the Q- and L-fractions in the peripheral nerve membrane cannot, of course, be decided until after an extensive analysis.

As far as the variations in the quick spike fraction are concerned we will in this connection only draw attention to the fact that — as appears from Fig. 13 — the change in spike peak level is much more pronounced than the change in the quick repolarization level during the natural variations in membrane potential level observed in our experiments. There are certain interesting analogies with the changes of the Q-fraction in nerve during repetitive nerve stimulation but in view of the more complex building-up of the action potential recorded from the soma of spinal neurons it is not for the present possible to go into a detailed discussion (cf. General comments).

The quick repolarization level is approximately the same during natural activation and electrical ortho- or antidromic stimulation, indicating a similar spike process in the various modes of activation. The potential configurations illustrated in Fig. 12 are characteristic, with a repolarization level exceeding the initial membrane potential level when this is low, whereas at a high membrane potential value the quick repolarization level does not attain the pre-spike level. Similar effects have been demonstrated also in interneurons (HAAPANEN, KOLMODIN and SKOGLUND 1958). That these phenomena reflect certain general properties of the nerve membranes is evidenced by the observation that a similar reversal of the afterpotentials can be induced in myelinated

nerve fibres by artificial polarization (*e. g.* SCHAEFER 1940, CORABOEUF and BOISTEL 1956).

As has been shown in Fig. 9, the amplitude and duration of the *slow* repolarization phase may vary considerably even when the membrane potential level is practically unchanged, and this suggests that it represents a more complex process than the "hyperpolarization" phase after an antidromic impulse, whose configuration is constant for a given type of neuron and at a given membrane potential value (cf. ECCLES 1957). A systematic comparison of the slow repolarization phases during artificial and natural activation might reveal out of which potential components the slow repolarization phase during sustained activity is composed. For the present, this phase may *a priori* be presumed to be the result of an interaction between the rhythmical membrane oscillations discussed above and genuine spike afterprocesses. The fact that a typical slow repolarization phase may appear without a preceding recordable spike indicates that the spike afterprocess cannot be the predominant component. The slow post-spike repolarization phase as a link in rhythmical membrane oscillations will be further discussed in Section III.

The slow repolarization phase is chiefly characterized by its considerable decrease in amplitude parallel with an increase in the frequency of the sustained discharge; this can best be seen from the diagram Fig. 5. These changes in the potential configuration are strikingly similar to those of the sensory cell, as illustrated in Fig. 6 *D* in EYZAGUIRRE's and KUFFLER's work (1955).

From the point of view of the main theme of this work, the changes in the slow potential fraction are of great interest. Considering the role played by the L-fraction for the initiation of rhythmical activity in peripheral nerve and ganglion cell soma it is tempting to assume that, in spinal neurons, the decrease in membrane potential value up to firing level for sustained activity chiefly represents changes in a slow fraction of similar type.

A comparison between different cells shows that the size of the slow potential fraction — in terms of maximal range of slow de- and repolarization as indicated in Fig. 13 — may vary considerably in relation to the total membrane potential value. On the basis of the limited material at hand so far it is not possible to decide to what extent accidental factors, such as the position of the electrode, cell injury, variations in the state of the preparation in regard to oxygenation, temperature etc., may play a role, or if the dissimilarities observed in the size of the slow potential fraction reflect different membrane properties of different types of cells. We have noticed a certain tendency of the slow fraction to be relatively smaller in interneurons than in motoneurons, which is of interest in so far as the former type of cells has marked repetitive properties, but these differences should be judged with caution; one apparent source of error is variations in the pre-existent activity impinging on the neuron causing varying degrees of active depolarization.

LAPORTE and LORENTE DE NÓ (1950) showed that the repetitive "d"-type of postsynaptic discharge in the toad ganglion can be attributed to

a reduced value of the L-fraction and thus forms a counterpart to the repetitive discharge induced in peripheral nerve by a catelectrotonus. The synchronous "s"-type of postsynaptic response, on the other hand, may be initiated without removal of more than a small part of the L-fraction and is thus analogous to the nerve response elicited by a brief pulse of a cathodal current. The authors emphasized this dual nature of the synaptic transmission in sympathetic ganglia and also pointed out the analogy existing between these "s"- and "d"-responses, on one hand, and the monosynaptic response and the more long-lasting type of "after-discharges" in the spinal cord, on the other hand (cf. LLOYD 1955).

The present series of experiments has given some further support to the concept that the slow membrane potential change in sustained discharges may represent an excitation mechanism, different from that of single-spike responses to a synchronous volley. From Fig. 12 *C* it appears that, using synchronous afferent stimulation, it is possible to elicit an impulse at a higher membrane potential level than the natural firing level for the onset of sustained discharges. In analogy with the findings quoted above, this might indicate a type of excitation mechanism which does not require removal of the slow fraction to such an extent as is necessary for rhythmical activity. It is also noteworthy in this connection that in certain preparations — often such in less good state — it is possible to elicit single responses to synchronized volleys, whereas no sustained responses can be evoked; an obvious analogy to these results is to be found in the observation made by BERNHARD and SKOGLUND (1947) that slow ventral root potentials were only obtained from preparations in good condition, whereas monosynaptic responses did not require preparations in the same good state. However, other explanations of these phenomena can also be found, *e. g.* along the lines of differences in accommodation properties of the cell membranes.

II. Membrane potential changes during inhibition.

1. Repolarization caused by natural activation of inhibitory sources.

As already mentioned, BARRON and MATTHEWS (1938) showed that the application of an inhibitory stimulus results in a fall of the ventral root potential level maintained by an excitatory stimulus and that this potential level is built up again after the inhibitory stimulus is discontinued. By means of the intracellular recording technique it is possible directly to demonstrate the corresponding membrane potential changes of the motoneuron soma. In Fig. 16 the dotted line represents the highest membrane potential value, 82 mV, observed in a given motoneuron in non-firing state. By changing the proprioceptive inflow, the potential level of this motoneuron could be brought to the threshold of firing, 76 mV, resulting in a very slow, intermittent discharge. By application of

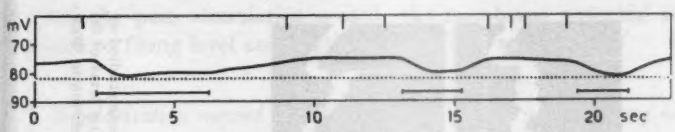

Fig. 16. *Motoneuron.* Repolarization by inhibitory natural stimulation. *Dotted line* at 82 mV represents highest membrane potential value recorded in non-firing state. Proprioceptive stimulation keeps neuron depolarized to firing level of 76 mV except during periods of simultaneous exteroceptive stimulation (ipsilateral pad pressure marked by *horizontal lines*). Impulses marked at top of figure. Drawing from original cathode ray recording.

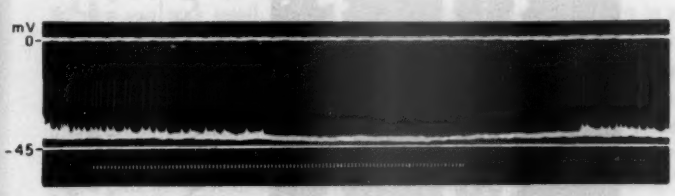

Fig. 17. *Interneuron.* Repolarization by inhibitory stimulation. *White line* at 45 mV represents highest membrane potential value recorded in non-firing state. Proprioceptive stimulation (stretch of ipsilateral gastrocnemius muscle maintained throughout the record) keeps neuron depolarized to 40 mV, which causes firing at 32 per sec at beginning of record. Exteroceptive stimulation (*dotted line*) results in lowering of membrane potential level and cessation of discharge. *Time bar* 100 msec.

pressure on the ipsilateral pad the membrane potential could be brought back towards its original level, and the discharges then always stopped. After cessation of the pressure stimulation the membrane potential again returned to 76 mV. The figure shows three inhibitory pad pressure stimulations of this kind, of different strength and duration, resulting in slow potential shifts of varying shape.

The same process of repolarization by inhibitory stimulation can also be demonstrated in interneurons. In the example shown in Fig. 17, the highest membrane potential value recorded was 45 mV. By stretch of the ipsilateral gastrocnemius muscle this neuron could be brought to discharge at a rate of maximum 32 per sec, the firing level then being 40 mV. In this interneuron application of pain stimulation on a certain area of the tail was observed to result in a slowing-down and finally a complete cessation of these discharges, as appears from the figure; simultaneously the membrane potential was brought back to the vicinity of the original level.

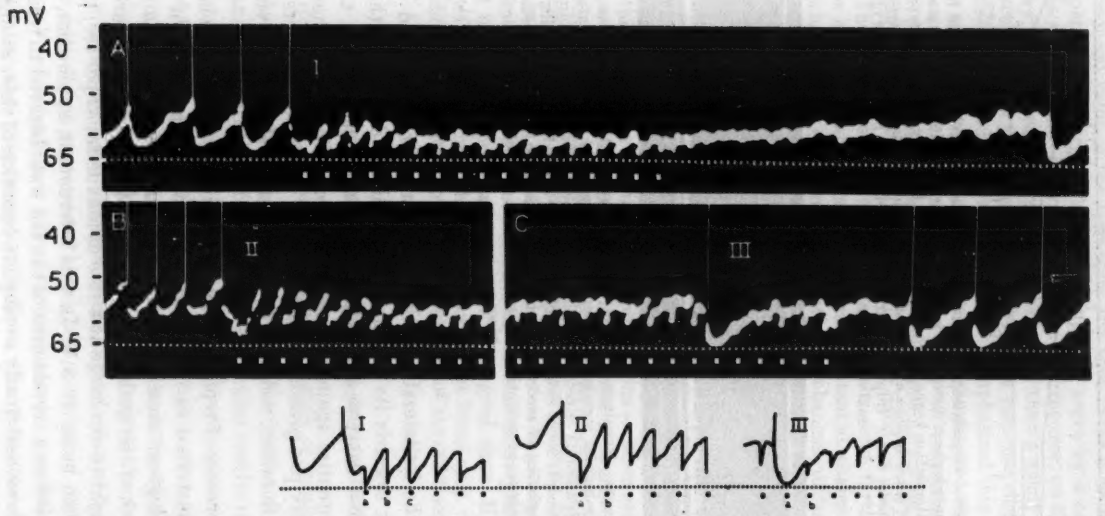

Fig. 18. Same motoneuron as in Fig. 3. Repolarization effects by repetitive electrical stimulation of inhibitory afferents studied at low (*A*) and high (*B*) initial activity levels. Full explanation in text. *Thin dotted line* at maximum spike repolarization level of 65 mV. At *large white dots* sural nerve stimulation of constant strength. Diagrams I, II and III below records are schematic drawings of corresponding parts of the records at higher magnification. *Time bar* 100 msec.

When the pain stimulation ceased, the membrane potential returned to firing level and rhythmical activity set in again.

2. Repolarization caused by artificial stimulation of inhibitory afferents.

In another series of experiments inhibitory effects in motoneurons produced by synchronous afferent volleys in skin afferents have been studied (cf. ECCLES 1953). In the experiment to be described (Fig. 18), repetitive activation of the sural nerve at a rate of 26 per sec was used. Series of inhibitory volleys were set in at different activity levels of the motoneuron. At the beginning of record *A* the motoneuron has a comparatively low discharge frequency of 11 per sec. The first inhibitory impulse volley reaches the neuron at the height of a slow post-spike repolarization phase (cf. also diagram I); it results in a polarization effect of only a few mV and can be seen in the shape of a small dip (I*a*) to the 65 mV level marked by the dotted line. The next inhibitory volley sets in when the following depolarization phase has reached about halfway to firing level and the membrane potential is then once more brought back (I*b*) close to the 65 mV level, corresponding to a repolarization effect in this case of 6 mV. The next depolarization phase has reached close to firing level when it is once more reduced — this time by about 8 mV — by the next inhibitory impulse (I*c*). In the following sequence of events, however, the depolarization phases attained between each inhibitory stimulus gradually become smaller and soon a steady state is reached, corresponding to a fairly constant membrane potential level of about 57 mV, from which the inhibitory effects can be observed as dips to the 65 mV level, at the dotted line. This experiment clearly demonstrates how the inhibitory stimulus tends to bring the membrane potential back to a given level. After cessation of the inhibitory stimulus the membrane potential level slowly approaches the firing level and after some characteristic oscillations the first spike appears.

In record *B* inhibitory stimulation of the same strength and frequency as in *A* has been applied to the motoneuron when at a higher activity level, corresponding to a discharge frequency of 20 per sec. Also in this case (cf. diagram II) the first inhibitory stimulus happens to set in at the height of a repolarization phase and it leads to an initial maximal dip (II*a*) reaching close to the 65 mV level. When the next stimulus sets in the membrane potential is

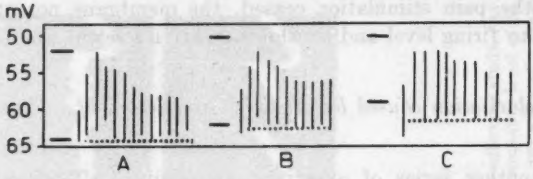

Fig. 19. Diagram of inhibitory repolarization effects (*vertical lines*) at various initial activity levels (*A*, low; *B*, medium; *C*, high activity). *Horizontal bars* indicate firing and repolarization levels before inhibition. Average final repolarization level marked by *dotted lines*. See text.

very close to the firing level and the result is a dip of 10 mV (II*b*). Gradually a steady state is attained between the depolarizing and the repolarizing influences, in the same way as in record *A*, although this time at a distinctly lower level.

In this experiment the inhibitory stimulus was maintained for more than 10 sec and during this period the steady state level could be seen gradually to attain the firing level of 53 mV (beginning of record *C*). As a consequence a spike was elicited in spite of the maintained inhibitory stimulation.

It is also of interest to note (cf. diagram III) that an inhibitory stimulus setting in at the height of the slow repolarization phase after this spike causes no further downward dip (III*a*), while the effect is only just discernible halfway up on the depolarization phase (III*b*); when the steady state level has been reached the dips are clearly seen again.

The successive lowering of the average inhibitory repolarization level with increasing rate of activity appears from Fig. 19, in which the repolarization effects at maximum and minimum activity levels, as observed in the two records of Fig. 18, have been schematically depicted (*A* and *C*), as also the effects at medium activity level (*B*). The diagrams show that in the actual experiment the inhibitory repolarization dips never exceeded 65 mV, corresponding to post-spike repolarization at the lowest discharge frequency, but that they might very well be below the raised post-spike repolarization levels at higher discharge frequency, as appears from diagrams *B* and *C*.

Sweep pictures showing the initial time course of the inhibitory repolarization by exteroceptive stimulation are given in Fig. 20. The records, obtained from the same experiment as in

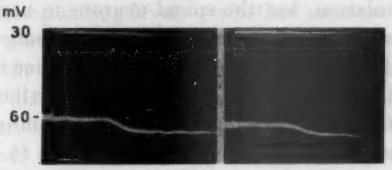

Fig. 20. *Motoneuron.* Repolarization effects of single shock stimulation of sural nerve, studied on fast time scale. Initial membrane potential level in *A* 60 mV; in *B*, 62 mV. *Time* in msec.

Fig. 18 and referring to an adjacent motoneuron with similar excitability properties, show that with the sural nerve stimulation used the latency is about 4 msec and that the repolarization is completed in 2 msec. The larger amplitude of the repolarization at lower initial value of the membrane potential (record *A*; cf. *B*) — in agreement with the results of Fig. 19 — can also be seen.

Discussion.

The experiments described in this section have only been designed to elucidate some special aspects of the wide problem of spinal inhibition. Only exteroceptive inhibitory stimulation has been used and the effects have been studied mainly on initially depolarized neurons. No attempts have been made to determine the degree of complexity of the synaptic connections, *e. g.* by latency determinations (LLOYD 1943); as far as the motoneurons are concerned direct connections with exteroceptive afferents can at any rate be ruled out.

The experiments shown in the first part of the section illustrate the very slow variations in the membrane potential level that can be brought about by means of natural activation of inhibitory sources; the motoneuron recording, Fig. 16, is an example of changes chiefly in the sub-firing range, whereas the interneuron recording, Fig. 17, exemplifies an inhibitory counteraction of a more pronounced depolarization corresponding to a relatively high initial discharge frequency. The membrane potential changes shown in the latter case are accompanied by the typical frequency variations previously studied in detail by KOLMODIN (1957).

The changes illustrated in these experiments have been induced

by active stimulation, but the spinal neurons in non-anesthetized preparations are no doubt continuously being subjected to both excitatory and inhibitory influences which balance the membrane potential level in an analogous way. It is thus obviously extremely difficult to determine a "resting" value of the membrane potential. In the present studies we have not been able to establish any inhibitory increase of the membrane potential value beyond the maximum value recorded in the single experiments, but even if such an increase should have been observed this need not necessarily imply an actual hyperpolarization but may still represent a counteraction of pre-existent depolarization due to impinging activity.

The experiments with artificial stimulation of inhibitory afferents shown in the second part of the section are of special interest in so far as they allow of a quantitative study of the inhibitory effects during natural variations of the membrane potential level. The neurons have been in a state of rhythmical firing when the inhibitory stimuli have set in, and there is a close similarity between this experimental situation and some experiments by KUFFLER and EYZAGUIRRE (1955) on the sensory cell of crayfish, in which the effects of single inhibitory volleys on membrane potential oscillations induced by natural stimulation were studied. In spite of the widely different organization of the two types of nerve cells, the potential pictures obtained show a striking resemblance. In both cases the amount of repolarization by the inhibitory impulse is greatest when the depolarization is largest, and at low frequencies of the inhibitory stimulation a redevelopment of depolarization occurs between each inhibitory stimulus. KUFFLER and EYZAGUIRRE found that at higher frequencies of inhibitory stimulation the membrane potential was held steady at a certain level. The same phenomenon has been observed — although not illustrated — in the present investigations and, besides, a direct analogy can no doubt be found in the smooth shifts of the membrane potential during natural inhibitory stimulation as a result of a disperse inflow of high frequency.

In the sensory cell an equilibrium level has been shown to exist, at which the inhibitory stimulus does no longer elicit any repolarization effect and below which inhibitory *de*polarization effects may occur. The equilibrium level most frequently observed was 5 mV below the resting potential level, the "true" value of which is easier to determine in these cells. It is interesting that in

Fig. 18 the corresponding level — whether measured as the maximal level of the inhibitory repolarization dips in record *A* or measured as the level where the inhibitory repolarization effect is no longer discernible in the post-spike repolarization phase (in *B*) — is at, or just below, the maximal membrane potential value recorded, 65 mV. We have not observed any inhibitory *de*polarization effects in spinal neurons within the physiological range of the membrane potential variations studied, and according to KUFFLER and EYZAGUIRRE it seems to be doubtful whether, under normal conditions, such a mechanism is active in the crayfish cell. In their analyses of inhibitory postsynaptic potentials of motoneurons COOMBS, ECCLES and FATT (1955) found a reversal to depolarizing response when the membrane was hyperpolarized about 10 mV beyond resting level. Since these analyses are based on an essentially different experimental procedure, a comparative discussion is, however, hardly profitable at the present stage.

It appeared from Fig. 19 that the inhibitory stimulus tends to bring the membrane potential down to a given level which varies with the initial state of activity of the cell. With long-lasting stimulation a successive change of this level may occur which might be interpreted as an adaptation to the inhibitory stimulus. It is hardly to be expected that satisfactory explanations of these and other phenomena can be found without more precise data on the presynaptic organization of the cells investigated.

The experiments described in this paper do not provide any information of the closer mechanism of the soma membrane behind this inhibitory counteraction of depolarization. Besides processes of interaction on the soma or dendrites of the neuron recorded from, there are also other mechanisms to be taken into consideration. Similar repolarization changes in the soma may be produced if the amount of excitatory impulses impinging on the neuron is reduced by blocking at an earlier relay station. This possibility must always be considered when an interneuron is interconnected in the pathway, but interaction at the level of primary terminals is also to be taken into account. The conclusion drawn by BARRON and MATTHEWS (1938) that "the inhibitory stimulus cannot be reducing the (depolarization) potential by an opposing process but is preventing its development" may thus still be valid.

III. Miniature potentials and their variations with changes of the afferent inflow and of the membrane potential level.

The occurrence of small baseline oscillations of less than 1 mV in motoneurons was first described by BROCK, COOMBS and ECCLES (1952) who presumed that these miniature potentials were attributable to random synaptic bombardment ("synaptic noise"). In connection with the description of Figs. 3 and 6 above it was pointed out that the small baseline oscillations occurring in the motoneuron recorded in these figures could be seen to increase when the membrane potential approaches the firing level, and in the following further examples will be given of the variations of these miniature potentials with changes in the excitability state of the neuron.

An illustration of this phenomenon in interneurons is given in Fig. 14, record *A*, which shows how natural activation (by pad pressure) causes a definite increase in the baseline oscillations preceding the firing of the first spike potential. From record *E* it appears that the increased baseline oscillations are also maintained for some time after cessation of the spike discharge, as a result of the gradual decrease in afferent inflow upon release of the pad pressure.

Such low-amplitude potential variations may be difficult to exhibit on records taken with ordinary amplification, but they often appear quite clearly in the form of variations in the noise level in the loudspeaker connected with the output stage, and during natural activation of different peripheral sources this noise level has frequently been observed either to increase or decrease.

In some neurons the miniature potentials had a considerable amplitude, possibly due to a favourable electrode position, and such cells are well suited for an analysis of these phenomena. The interneuron studied in Fig. 21 was of a common convergence type (cf. KOLMODIN 1957) which could be influenced both from an exteroceptive and a proprioceptive source. This particular neuron was also especially easy to analyse because its miniature potentials could be studied undisturbed by the potential fluctuations accompanying natural spike discharges, as the neuron could not be brought to fire any spikes, whether owing to insufficient peripheral activation from the sources involved or to a damage of the neuron is not possible to say.

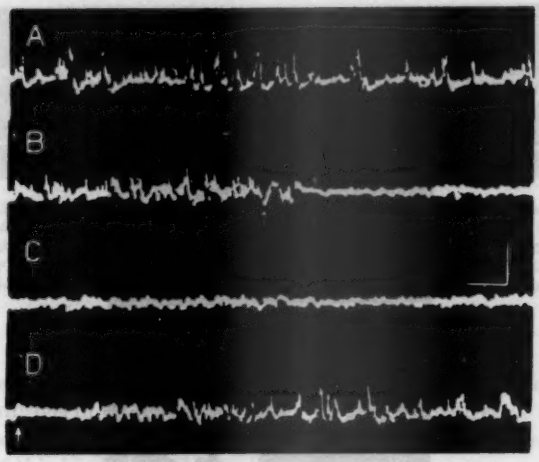

Fig. 21. *Interneuron.* Miniature potential variations with changes in afferent inflow. *A*, maximal activity caused by stretch of ipsilateral gastrocnemius muscle. Continuous recording throughout *B*—*D*. *B*, resting activity in absence of active stimulation. At *arrow* in *B* application of pad pressure which is maintained throughout *C*; in *D*, at *arrow*, cessation of pad pressure. *Time bar* 200 msec. *Vertical bar* 5 mV.

When no afferent stimulus was applied, the amplitude variations of the miniature potentials had a range of about 4 mV (see record *B*). After application of stretch to extensor muscles of the ipsilateral hindlimb the potentials increased considerably and had a certain tendency to intermittent synchronization (*A*). On the other hand, the pre-existent activity could be virtually abolished by application of pad pressure (see records *B* and *C*). On relaxation of this pressure the "normal" miniature potential activity was resumed (*D*).

The shape of these potentials can be studied more closely in the sweep pictures of Fig. 22, taken on a fast time scale and at various activity levels. Record *A* shows the oscillations of very low amplitude still present during inhibitory stimulation, and record *B* those during a phase of moderate activity in the absence of any stimulation. Record *C* illustrates typical oscillations of higher amplitude seen during activation of the proprioceptive source. The smallest potential fluctuations clearly discernible against the

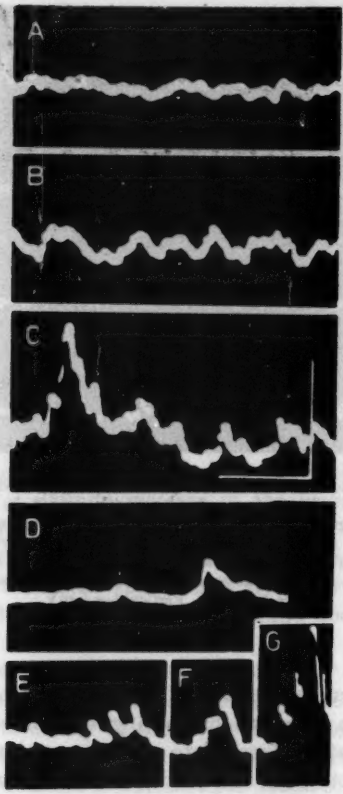

Fig. 22. Miniature potential variations from same interneuron as in Fig. 21 but on faster time scale. See text. *Time bar* 10 msec. *Vertical bar* 10 mV in *D*, in all other records 5 mV.

baseline have an amplitude of 1—3 mV (record *D*); they may be designated potentials of "unitary" type. The last series of records (*E*—*G*) shows potentials of higher amplitude, whose shape suggests that they are the result of a stepwise building-up of such unitary potentials.

Corresponding variations in the amplitude of the miniature potentials parallel with changes in the natural inflow can be ob-

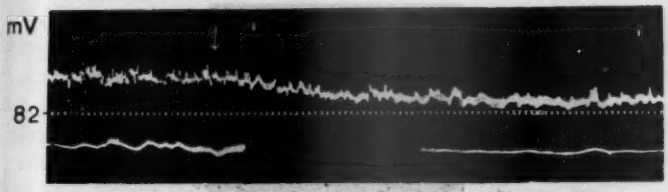

Fig. 23. *Motoneuron.* Variations in miniature potentials with membrane potential level. *Dotted line* at 82 mV, highest membrane potential value recorded in non-firing state. Change from firing level at beginning of record towards 82 mV level caused by ipsilateral pad pressure (at arrow). *Inset* records (below) on faster time scale of miniature potentials during depolarized and repolarized state.

served also in motoneurons, and in view of the more stable recording conditions in these cases a correlation to the changes in membrane potential level can more readily be made. Fig. 23 illustrates a study of this kind made, by the way, on the same motoneuron as in Fig. 16. As may be seen at the beginning of the record, miniature potentials of rather high amplitude could be observed in this motoneuron when at firing level, 76 mV. Application of pad pressure resulted in a downward shift of the membrane potential level, and simultaneously a distinct decrease of the miniature potentials could be seen; after cessation of the stimulation they increased again. Some sweep pictures taken at different periods (inset below) also clearly illustrate the reduced range of potential fluctuations in the repolarization period.

Also in rhythmically discharging neurons it can often be observed that the miniature potentials increase in amplitude as the membrane potential approaches the firing level. Thus, *e. g.*, it is apparent that in some of the spike intervals in Fig. 3 the miniature potentials are more pronounced in the upper than in the lower part of the depolarization phase.

By far the greater part of the miniature potentials may be characterized as depolarization potentials with a faster initial phase representing an inside change in positive direction, followed by a slower repolarization phase, only exceptionally reaching below the average baseline. However, even if so far found only occasionally, miniature potentials have actually been observed which might justify the designation of repolarization potentials, as constituting dips below the average level of baseline oscillations. When their amplitude is as small as in Fig. 24 *A* (arrows), they

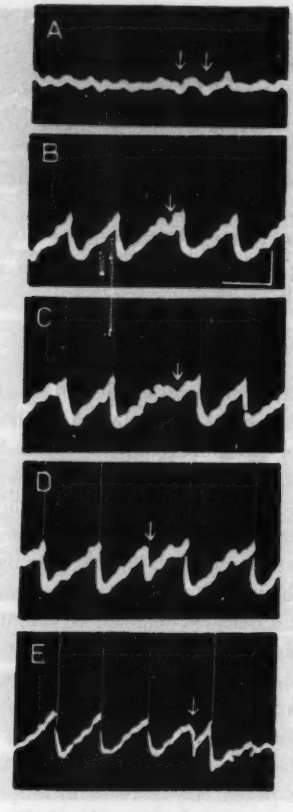

Fig. 24. ***Motoneurons.*** **Miniature potentials of repolarization type (arrows). *A*, in non-firing neuron; *B—D*, in firing neurons, causing prolongation of depolarization phase and disturbance of firing rhythm; *E*, inhibitory repolarization caused by stimulation of the sural nerve (arrow), for comparison. *Time bar* 100 msec. *Vertical bar* 10 mV.**

may not be very easily identifiable. That these small downward dips may actually be of functional significance is, however, evident from observations made when they set in on the oscillations in rhythmically discharging neurons, as in the following

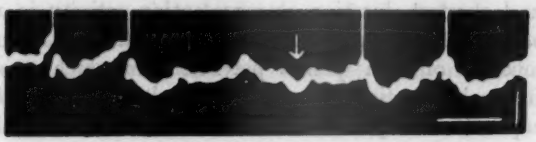

Fig. 25. *Motoneuron.* Isolated occurrence of repolarization potential (*arrow*) in phase with post-spike oscillations. See text. *Time bar* 100 msec. *Vertical bar* 5 mV.

records. The downward dip in *B* is small but causes nevertheless a definite prolongation of the depolarization phase, resulting in a slightly disturbed rhythm; the similar dip of longer duration in *C* brings about a still greater delay in spike firing, and a spike interval nearly twice as long as normal is produced by the large repolarization dip in *D*. Record *E* is included to show that a similar potential picture can be obtained by setting in (at arrow) an inhibitory impulse volley just before the depolarization phase reaches maximum.

Fig. 25 shows yet another example of such a downward dip (at arrow) appearing in a non-firing period of an intermittently discharging motoneuron. At first sight one may be inclined to regard it as a randomly occurring dip, but another alternative should also be discussed. The non-firing period corresponds to two spike intervals, and it is evident that the negative dip occurs just when a slow repolarization should have set in if the spike discharge had been going on continuously. This timing may of course be accidental but it also suggests that the dip may be a link in a rhythmical sequence of slow repolarization potentials.

Discussion.

The low-amplitude potentials described above under the designation of miniature potentials have displayed varying configurations, from smooth baseline oscillations to more distinct spike-like potentials, but characteristic of all of them is their spontaneous appearance and their increase or decrease with changes in the afferent inflow. Further investigations may perhaps provide evidence that the potentials are of various types, but at the present stage it seems reasonable to regard the potentials of lowest ampli-

tude and shortest duration as representing activity in unitary fractions of the membrane and larger potentials as resulting from a summation of such quanta potentials, in which case variations in synchronization may of course result in all kinds of potential shapes. If some patches of the cell membrane are active and others inactive, it is obvious that a given electrode position in the cell may be more or less favourable for the recording of these potentials.

We have seen examples of unitary potentials summating to potentials of up to 10 mV amplitude and 20 msec duration, and it might be possible that the slow inter-spike oscillations are built up in such a quantal fashion. In several respects there is a great similarity to miniature end-plate potentials as described by FATT and KATZ (1952). The changes in end-plate activity produced by artificial presynaptic polarization (CASTILLO and KATZ 1954, LILEY 1956) are of a certain interest in view of our observation of variations in miniature potential activity parallel with changes in the membrane potential level.

Comparatively few observations have been made on repolarization potentials but there is no doubt that spontaneous miniature potentials of this kind exist, and this was perhaps to be expected as similar potentials can be elicited by electrical stimulation of inhibitory nerves. When we are now taking up these potentials for a more detailed discussion in spite of the relatively limited experimental data, the reason is some interesting analogies found in investigations on *Aplysia* cells.

By means of intracellular recording from certain types of *Aplysia* cells, ARVANITAKI and CHALAZONITIS (1957 a, b) could record, beside an autorhythmical prepotential activity of ordinary type, rhythmical potentials of opposite sign ("positive" potentials in their nomenclature) and of an independent frequency which decreased with increasing depolarization of the cell. The spike potentials and the positive potentials had a mutual depressing action on each other which was determined by their succession in the sequence. They showed that when a positive potential appears between two spikes the interval is prolonged; this is in direct analogy with the events in our experiments as illustrated in Fig. 24 *B*, *C* and *D*, showing repolarization potentials interfering with inter-spike oscillations. If, in *Aplysia* cells, the positive potential appears just before a spike is being fired the result may be a reduction of the spike size or a drop-out of the spike; these po-

tential pictures resemble closely those obtained from moto- and interneurons (Figs. 8 and 15) when the depolarization phase fails to reach the firing level, and this raises the question whether also in this case there is a counteracting influence of repolarization potentials although these are not discernible.

It may evidently be worth while to undertake a closer study of the occurrence of miniature potentials of repolarization type in spinal neurons and try to determine to what extent they may contribute to slow inter-spike oscillations. It is from this point of view we have paid special attention to the isolated occurrence of a repolarization dip in phase with the rhythmical slow potential oscillations, such as illustrated in Fig. 25 (at arrow).

In this connection some further interesting similarities observed in the potential configurations of the *Aplysia* cells and spinal neurons may be worth mentioning, as they may throw some light on the nature of the slow post-spike repolarization phase of moto- and interneurons during sustained rhythmical activity. If, in *Aplysia* cells, the "negative" spike potentials and the positive potentials have the same frequency, the two potentials may be perfectly synchronized so that the slow positive potential can be seen as a direct continuation of the falling phase of the spike (ARVANITAKI and CHALAZONITIS 1957 b, Fig. 2 *E*); this results in a potential configuration that has a striking resemblance to the regular inter-spike oscillations in our experiments as illustrated in Fig. 3. When the synchronization is less perfect, the final phase of the quick repolarization can be seen as a distinct phase followed by the positive potential (their figure 2 *D*); the pictures thus obtained are very similar to those illustrated in Figs. 9 and 10 in this paper. These observations point to the possibility that dissimilarities in the shape of the spike repolarization phases in spinal neurons may partly be ascribed to differences in the synchronization of the various potential components involved.

Summary.

Slow membrane potential changes accompanying excitation and inhibition processes have been studied in spinal moto- and interneurons of spinal cats, using intracellular recording technique.

Section I. Long-lasting shifts in membrane potential level have been recorded in both moto- and interneurons during sustained rhythmical activity.

A closer study of the slow membrane potential changes and their relation to spike potentials has revealed fundamentally similar events in moto- and interneurons; the quantitative analyses have mainly been performed on motoneurons whose steady membrane potentials allow of studies for sufficiently long periods. Special attention has been given to the slow membrane potential oscillations during rhythmical firing.

The firing level for the onset of rhythmical spike discharges was found to be relatively constant in one and the same neuron; with increase in discharge frequency a definite lowering of the firing level occurs, an approximately linear relation being found to exist between firing level and impulse interval.

During periods of regular rhythmical firing subthreshold membrane potential oscillations may occasionally be seen associated with spikes of reduced amplitude or not accompanied by any recordable spikes.

The potential analysis has led to a tentative differentiation between an initial quick and a later slow phase of repolarization. The quick repolarization level, more or less marked in different neurons, is of approximately the same value during natural activation and single ortho- or antidromic stimulation; it varies within relatively narrow limits during the large natural variations in membrane potential observed in the experiments.

The slow repolarization level, which may show great variations even at one and the same membrane potential level, undergoes a pronounced reduction with increasing frequency of the rhythmical activity. The relation between the slow repolarization level and the impulse interval is found to be approximately linear.

The typical variations in quick and slow membrane processes during various activity levels of a motoneuron have been diagrammatically represented. The possibility is discussed that the quick spike processes and slow potential variations represent changes in different membrane potential fractions, analogous to the Q- and L-fractions established in peripheral nerve.

The evidences in favour of an inherent oscillation mechanism in the membrane activated at a critical level of depolarization and primarily responsible for the discharge frequency are discussed.

Section II. Membrane potential changes during inhibition have been studied in moto- and interneurons using exteroceptive inhibitory stimulation. In both types of neurons natural activation of inhibitory sources resulted in a slow repolarization not exceeding

the highest membrane potential recorded in the absence of excitatory stimulation.

The inhibitory effects of synchronized afferent volleys have been analysed in motoneurons in different states of rhythmical activity. The amplitude of the repolarization dip produced by each afferent volley was found to depend on the membrane potential level at the onset of inhibition. During repetitive stimulation a steady state was gradually attained, the level of which varied with the initial state of activity of the neuron.

Section III. Both in moto- and interneurons the occurrence of random miniature potentials of varying amplitude has been demonstrated, those of larger amplitude (10 mV) apparently being built up out of the smaller "unitary potentials" of 1—3 mV.

Increase as well as decrease of the miniature potential activity could be produced by natural activation of extero- or proprioceptive sources influencing the neuron. Correlations to the membrane potential level showed a reduced amplitude range of miniature potentials during increased membrane potential.

In addition to the most common type of miniature potentials representing an inside positive change, potentials of the opposite sign were occasionally observed. The functional role of such randomly occurring repolarization potentials has been exemplified and a comparison made with similar phenomena observed in other types of nerve cells.

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Activation of the Contractile Mechanism in Striated Muscle.

By

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The general tendency has been to consider a membrane potential change as an essential link in the activation of the contractile mechanism in muscle (KATZ 1950, HUXLEY 1957). In agreement with these concepts are the observations that an electric current produces a muscle contracture only when it simultaneously causes membrane depolarization (*e. g.* STEN-KNUDSEN 1954) and that a contracture produced by a drug is accompanied by a reduction of the potential difference across the membrane (KUFFLER 1946). In plain muscle, however, EVANS, SCHILD and THESLEFF (1958) have reported that drugs may activate the contractile elements without the mediation of membrane depolarization. The evidence for such an action was the observation that mammalian plain muscle retains its responsiveness to a number of drugs even when completely depolarized in a solution containing a high concentration of potassium ions.

The results reported in the present paper show that also in striated muscle drugs may activate the contractile elements without producing membrane depolarization. Experiments on frog muscle suggest that the alkaloid caffeine starts a process in the muscle membrane which causes muscular contracture. Evidence

will be presented showing that this drug effect is not mediated by changes in the resting potential or in the ionic permeability of the muscle membrane.

Methods.

The experiments were made, at about 22° C, on the isolated sartorius muscle of the frog Rana esculenta or in some cases on the m. ext. l. dig. IV.

Isotonic contractures were recorded on a slowly moving smoked drum with a light (0.8 g) frontal lever with a writing ratio of 1 : 5. The volume of the muscle bath was 5 ml.

Tension recordings were made with a sensitive strain-gauge of the Statham type (modified by Swema-Elema, Sweden) and photographed on a cathode-ray tube operated through a d. c. amplifier.

For local application of caffeine to single fibres the muscle was mounted on a transparent perspex block in a Ringer bath on a microscope stand. Caffeine was applied to an edge fibre of the muscle from a micropipette filled with 80 mM caffeine solution and having an external tip diameter of 2—3 μ. To facilitate the release of caffeine from the tip of the pipette an electric current or positive air pressure was in some instances applied to the pipette. The drug pipette was carried on a Zeiss slide micromanipulator. The position of the tip of the pipette and the drug effects were observed visually in a binocular microscope (Zeiss Standard GF 525) at 180 × magnification. Illumination was provided by passing polarized light up through the preparation. When the tip of the drug pipette was inserted into a muscle fibre its intracellular position was confirmed by the recording through the pipette of a negative potential change of 60—70 mV.

Measurements of the resting membrane potential and of the transverse membrane resistance (*i. e.* the "input" resistance) of single muscle fibres were made with intracellular electrodes as described by Fatt and Katz (1951).

The composition of the Ringer's fluid was 116 mM NaCl, 2 mM KCl and 1.8 mM $CaCl_2$. The K_2SO_4 solution was isotonic (95 mM). Aqueous solutions of caffeine (1, 3, 7-trimethylxanthine) containing 1.5×10^{-2} w/v of the free base were prepared for each day's experiment.

Results.

Contractile responses to caffeine.

Isometric and isotonic contractures: When a frog's striated muscle is immersed in Ringer solution containing $2.5—10 \times 10^{-4}$ w/v caffeine tension develops and the muscle contracts (Fig. 1 and 2).

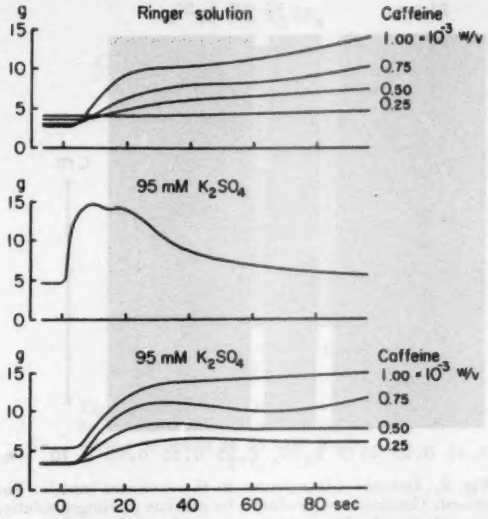

Fig. 1. Upper record: Isometric tensions produced by caffeine in a sartorius muscle immersed in Ringer solution. Middle record: The tension produced when the Ringer's fluid is substituted by 95 mM K_2SO_4. Lower record: Tensions produced by caffeine after 30 min immersion in K_2SO_4.

The contracture is sustained as long as caffeine remains in the bath and is fully reversible when the drug is removed. It is graded according to dose and can be obtained repeatedly without reduction over a period of several hours. With concentrations of caffeine above 10^{-3} w/v the muscle goes into a strong and irreversible contracture and removal of the drug no longer causes relaxation.

The maximum tension produced by caffeine in a concentration of 10^{-3} w/v in the sartorius muscle is about 12 g and in m.ext. l. dig. IV 2—3 g. These tensions are approximately one fifth of the tensions produced by tetanic stimulation of the muscle.

It is well known (Overton 1904) that a striated muscle when immersed in isotonic K_2SO_4 (or KCl) solution contracts rapidly and subsequently relaxes to about its initial length (Fig. 1 and 4). By exposure to 95 mM K_2SO_4 solution the resting potential of the muscle fibres is abolished or reduced to a small and uncertain value of a few mV in either direction (Castillo and Katz 1955).

Of particular interest in the present investigation was the obser-

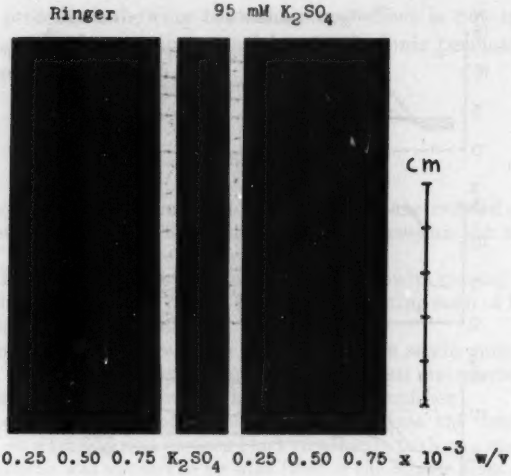

Fig. 2. Isotonic contractures in the sartorius muscle. First record: Contractures produced by caffeine in Ringer solution. Second record: The contracture produced by replacing the bath solution with 95 mM K_2SO_4. Third record: Contractures produced by caffeine in isotonic potassium sulphate solution.

vation that a muscle even when immersed in isotonic potassium solution responded to caffeine with a strong contracture. This is illustrated in Fig. 1 and 2 which show that concentrations of caffeine which produce contractures in Ringer solution are, at least to the same extent, effective when the muscle is soaked in isotonic K_2SO_4 (or KCl) solution. In fact it is evident from the records shown that the threshold for the caffeine effect is reduced in potassium. The period of time during which the muscle has been immersed in potassium solution is without influence on the caffeine effect. Fig. 3 shows isotonic contractures produced by caffeine in a muscle which has been soaked in 95 mM K_2SO_4 during 96 hours at + 4° C.

The rate of onset of the caffeine contracture is equally rapid in Ringer solution and in potassium sulphate solution. The maximum tension is reached within 20—50 sec. The rapidity of the caffeine effect is particularly obvious when the drug is applied rapidly to a thin muscle, see Fig. 4. The rate of recovery after removal of the drug is slower than the onset of a contracture but complete relaxation is generally achieved within 2—3 minutes.

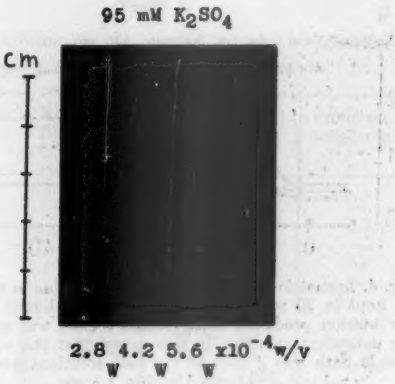

Fig. 3. Isotonic contractures produced by caffeine in a sartorius muscle soaked during 96 hours in 95 mM K_2SO_4 at + 4° C.

A few experiments have been made to determine whether Ca ions influence the caffeine effect. The addition of Ca up to five times its normal concentration or the depletion of Ca by soaking the muscle in 0.1 % versene (ethylenediaminetetraacetic acid tetrasodium salt) solution had, however, no observable effect on the contracture produced by a subsequent dose of caffeine. Methylated xanthines other than caffeine, *i. e.* theobromine and theophylline, produce qualitatively similar contractures in striated muscle but are less potent than caffeine.

Extra- and intracellular application of caffeine: When caffeine is allowed to diffuse from a micropipette to the outer surface of a muscle fibre a localized contracture develops over a distance of 30—50 μ. This effect can be obtained along the whole length of a fibre and is equally well observed in Ringer solution as in isotonic potassium. In no instance did local application of caffeine produce a conducted response in a muscle fibre.

The rapid development of maximum tension following the application of caffeine to a muscle bath suggested that the drug effect was mediated from the cell surface. It was, however, considered necessary to establish, by direct application of caffeine to the outside and to the inside of a muscle fibre, whether actually the caffeine effect was confined to the cell membrane. When caffeine

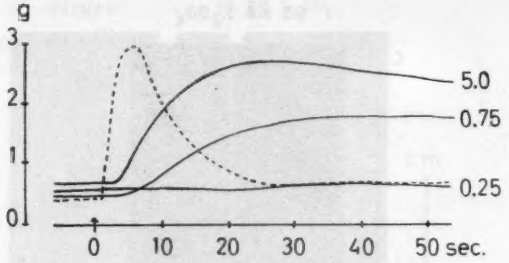

Fig. 4. Isometric tension produced by caffeine in m. ext. l. dig. IV kept in 95 mM K_2SO_4 solution. The dotted curve shows the tension produced when Ringer's fluid was substituted by the potassium solution. In this experiment the volume of the muscle bath was 2 ml and the bath solutions were completely changed within 1.5 sec.

dissolved in potassium solution was applied by ionophoreses from the tip of a micropipette (outer diam. $< 1\ \mu$) to the surface of a muscle fibre immersed in 95 mM K_2SO_4 solution a very localized form of muscle contracture was observed. Caffeine released by "catelectrotonic" current pulses of 10 msec. duration produced for instance a rapid twitch-like contraction involving only the adjacent two or three A bands. To release a sufficient amount of caffeine, however, strong electric currents had to be used and the ionophoretic method was for that reason considered unsuited for intracellular drug application. Experiments were therefore made in which caffeine was by diffusion alternately released extra- and intracellularly from a drug pipette, the drug effects being as previously visually observed in a polarizing microscope at 180 × magnification. When the tip of a drug pipette (outer diameter 2—3 μ) approached the muscle membrane, within a distance of about 5 μ, a localized contracture developed and when the tip was inserted into the fibre (its intracellular position being confirmed by the recording of a negative potential change of 60—70 mV) the contracture slowly disappeared. Upon withdrawal of the tip to the outside of the membrane the contracture redeveloped. These observations are strongly favouring the alternative that caffeine produces activation of the contractile mechanism by an action on the muscle membrane.

Table I.

Effects of caffeine on the resting membrane potential of single muscle fibres in Ringer solution.

Concentration of caffeine (mg/ml)	Number of fibres	Mean resting membrane potential (mV)	Produced tension (g)
Control	19	84.0 ± 1.1[1]	—
0.5	21	85.3 ± 1.6	2.5
Control	12	90.3 ± 1.9	—
0.9	11	88.6 ± 2.6	8.7
Control	15	91.3 ± 1.0	—
1.1	15	79.5 ± 1.8 $p = < 0.001$	17.5
Control	17	93.1 ± 2.2	—
1.3	12	76.6 ± 3.5 $p = < 0.001$	17.5

[1] S. E. of mean.

Effects of caffeine on the electric properties of the muscle membrane.

Resting membrane potential: Intracellular recording has shown that caffeine in concentrations below 10^{-3} w/v has no effect on the resting membrane potential of single muscle fibres (Table I). In concentrations producing an irreversible contracture, *i. e.* above 10^{-3}, caffeine causes a depolarization by 10—20 mV.

The results shown in Table I were obtained at a time when the maximum tension was reached, *i. e.* about 2—3 minutes after the addition of caffeine to the muscle bath. Measurements have also been made shortly (< 1 min) after the application of caffeine. Though these recordings sometimes were difficult to interprete because of movements caused by the developing contracture they nevertheless indicated that neither at this early stage of the caffeine effect, did any gross changes in the membrane potential occur.

When the muscle was immersed in isotonic K_2SO_4 no potential difference could be recorded across the muscle membrane and this state was unchanged by the application of caffeine.

Transverse membrane resistance: The electric resistance from a point inside a muscle fibre to the outside medium is not conspicuously altered by caffeine. This is shown in Table II in which values for the "input" resistance of fibres are given in Ringer and in 95 mM K_2SO_4 solution before and after the addition of caffeine

Table II.

Effects of caffeine on the transverse membrane resistance of muscle fibres immersed in Ringer and in 95 mM K_2SO_4 solution.

Fibre no.	Before addition of caffeine		After addition of caffeine 0.6—0.75 mg/ml		Recorded tension (g)
	Membrane potential (mV)	Transverse membrane resistance (KΩ)	Membrane potential (mV)	Transverse membrane resistance (KΩ)	
In Ringer's solution					
1	90	170	90	210	8.0
2	90	210	80	150	10.0
3	95	180	80	110	10.0
4	95	170	80	190	10.0
5	90	220	90	210	10.0
6	95	190	95	160	6.0
7	95	210	90	180	6.0
8	95	360	75	320	9.0
9	90	260	75	230	8.0
10	90	360	80	330	6.0
Mean	*92.5*	*233*	*83.5*	*209*[1]	*8.3*
In 95 mM K_2SO_4					
11	0	140	0	100	11.0
12	0	120	0	90	10.0
13	0	110	0	100	5.0
14	0	170	0	130	8.0
15	0	210	0	210	6.0
16	0	150	0	120	5.0
17	0	160	0	130	5.0
18	0	230	0	160	3.0
Mean	*0*	*160*	*0*	*130*[1]	*6.6*

[1] The slight reduction in the transverse membrane resistance after addition of caffeine to the muscle bath is statistically significant at the one percent level.

to the muscle bath. The kind of records from which the values were obtained are shown in Fig. 5. It is evident from Fig. 5 that caffeine does not affect the time course of the electrotonic potential and that it therefore is unlikely that the electric capacity of the membrane is changed. The rectifier properties of the muscle membrane in potassium solution (KATZ 1949) were maintained in the presence of caffeine.

The immediate effects of caffeine on membrane resistance are shown in Fig. 6. In that experiment electrotonic potentials were recorded at the time when caffeine was applied from a micro-

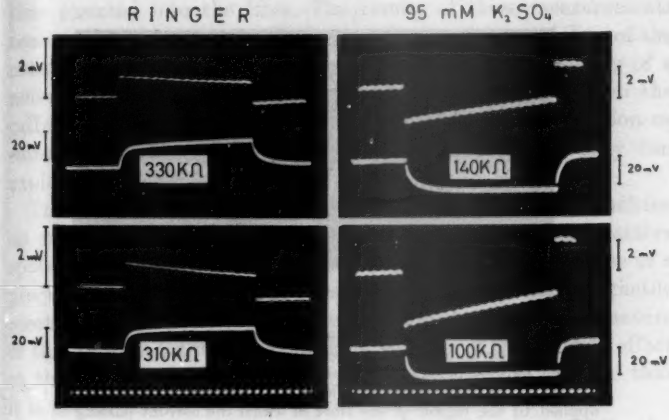

Fig. 5. Left side: In Ringer solution. "Anelectrotonic" potentials in a single muscle fibre (lower trace) and the applied rectangular current pulse. Upper record before and lower record 3 min after the addition of caffeine 7.5×10^{-4} w/v to the muscle bath. The resting membrane potential of the fibre was 90 and 80 mV respectively. Right side: In 95 mM K_2SO_4. "Catelectrotonic" potentials, upper record before and lower record after the addition of a similar concentration of caffeine. The transverse membrane resistance is shown for each fibre and measurement. Time scale 50 cps. (The current pulses were recorded by an a. c. pre-amplifier with a decay time constant of 0.9 sec at 0.37 maximum height, hence their non-rectangular shape.)

pipette to the muscle membrane in the immediate vicinity of the current passing and the recording electrode. It is obvious that no pronounced changes in the membrane resistance occurred. The depolarization which is observed was probably an artefact produced by movements during the developing contracture.

Discussion.

It has been known for a long time that caffeine produces a contracture in striated muscle and similarly has its stimulatory action on muscle metabolism been extensively studied (see review by Gasser 1930). It is, however, as shown in the present investigation, interesting that the drug effect, although presumably produced at the cell surface, is not mediated by a change in the resting potential or in the ionic permeability of the muscle membrane.

The experiments with extra- and intracellular application of

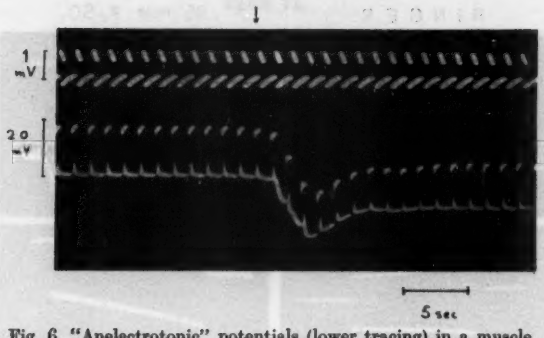

Fig. 6. "Anelectrotonic" potentials (lower tracing) in a muscle fibre in Ringer solution. The upper tracing shows the rectangular current pulses (for an explanation of their non-rectangular shape see Fig. 5). At the arrow an 80 mM solution of caffeine was locally applied to the region of the fibre in which the current passing and the recording electrodes were inserted. Note that caffeine does not alter the amplitude of the electrotonic potentials.

caffeine to a muscle fibre indicate that the drug produces a contracture only when applied to the outside of a fibre but not when released to the interior of the cell. This observation in combination with the finding of a rapid time course for the development of the caffeine contracture suggest that the drug effect is confined to the muscle membrane. To establish with certainty that the site of drug action is at the muscle membrane, a method has to be used which allows an estimate of the amount of drug released respectively extra- and intracellularly from the micropipette. This is, however, technically difficult and has not been attempted in the present investigation.

The absence of depolarization when caffeine is applied to a muscle and the equal effectiveness of the drug irrespective of whether it is added to a muscle immersed in Ringer or in isotonic potassium solution shows that a membrane potential change is neither produced nor necessary for the mediation of the caffeine effect.

Neither in Ringer solution nor in 95 mM K_2SO_4 did caffeine produce marked changes in the transverse membrane resistance. The slight but significant reduction in the transverse membrane resistance after the application of caffeine is presumably, since the membrane potential was also lowered, due to membrane damage produced by the microelectrodes when they were for the second

time inserted into the fibre. The results of these measurements consequently render it unlikely that the ionic permeability of the muscle membrane is markedly altered by the drug. Soaking of a muscle during 96 hours in 95 mM K_2SO_4 did not diminish the caffeine effect. This indicates that a selective flux of an ion or substance into the fibre from the outside medium hardly can explain the activation of the contractile mechanism.

The most likely explanation for the stimulatory effect of caffeine on muscle is that the drug by combining with certain "active spots" or "receptors" in the cell membrane causes the release of a process which by inward propagation activates the contractile mechanism. No indication has been obtained regarding the nature of this process (or processes) linking the site of the caffeine effect to the contractile elements but the present results indicate that it is not an electric current or potential change.

The reversibility of the caffeine contracture and the finding by HARTREE and HILL (1924) that the amount of heat released by caffeine in a muscle closely corresponds to the amount liberated by tetanic stimulation suggest that the contractile response in both instances has the same mechanism. It is consequently possible that the activation process which is started by caffeine in the membrane is the same as that triggered by the action potential.

Summary.

1. When a frog's striated muscle (m. sartorius) is immersed in Ringer solution containing $2.5—10 \times 10^{-4}$ w/v of the free base caffeine tension develops and the muscle contracts. The contracture is graded according to dose and reverses rapidly upon removal of the drug. Higher concentrations of caffeine produce an irreversible contracture.

2. In a muscle immersed in isotonic K_2SO_4 or KCl solution caffeine produces exactly the same kind of muscle contracture as in a muscle kept in Ringer's fluid. Prolonged soaking (96 hours) of a muscle in isotonic K_2SO_4 solution does not reduce its responsiveness to caffeine.

3. Local application of caffeine from a micropipette to a muscle fibre produces a localized contracture when the tip of the pipette is at the outer surface of the cell membrane. When the tip is inserted into the fibre no contracture is observed indicating that the caffeine action is confined to the muscle membrane.

4. In concentrations below 10^{-3} w/v caffeine does not alter the resting membrane potential of muscle fibres. Neither does the drug markedly reduce the transverse muscle membrane resistance.

5. It is suggested that caffeine at the muscle membrane starts a process which activates the contractile mechanism in the interior of the fibre. This process is believed to be the same as that activated by the propagated action potential. The nature of the process (or processes) linking the membrane to the contractile elements is unknown but the present results makes it unlikely that it is an electric current or potential change.

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Effect of Pyridoxine Deficiency on Cholesterol and Polyenoic Fatty Acids in Chicks.

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Several authors have noticed an influence of pyridoxine on the metabolism of cholesterol and polyenoic fatty acids. Thus, MEDES and KELLER (1947) found a higher content of polyenoic fatty acids in the tissues of fat-deficient rats when the diet contained pyridoxine. Later, WITTEN and HOLMAN (1952) studied the effect of feeding 1 mg pyridoxine and/or 100 mg ethyl linoleate and 100 mg ethyl linolenate, respectively, to severely acrodynic rats. They found that both pyridoxine and linoleate had a stimulating effect on growth and fat synthesis and produced an increase in the total amount of arachidonate. Linolenate plus pyridoxine also stimulated growth and fat synthesis, and caused an increase in hexaenoic acid. It was suggested that pyridoxine may act as a coenzyme for enzymes responsible for the observed effects (HOLMAN 1954). The relationship between plasma-cholesterol and pyridoxine-deficiency in Rhesus monkeys fed low fat diets containing 1 % cholesterol has been examined by GREENBERG and RINEHART (1951) who found the greatest hypercholesterolemia in the pyridoxine deficient monkeys. COMBRIDGE (1956) made histological examinations of certain organs of pyridoxine deficient rats

and found heavy lipid infiltrations in the centrilobular region of the livers of severely deficient rats.

The present paper describes the effect of pyridoxine deficiency on cholesterol in plasma, liver, heart, and aorta, and on polyenoic fatty acids in heart and liver of chicks fed fat-free diets or diets containing 10 % peanut oil with and without 1 % dietary cholesterol.

Experimental.

The experiments were carried out in two sections. The first section (A) comprised 8 groups with 10 chicks in each. Four of these groups (groups 1785, 1787, 1789, 1791) received a "normal" commercial diet (DAM *et al.* 1957)[1] during the first two weeks after hatching; thereafter they were given the experimental diets nos. 1785, 1787, 1789, and 1791, respectively, for 4 weeks (see Table I). The other four groups (groups 1786, 1788, 1790 and 1792) received the experimental diet no. 1785 from hatching until the fourteenth day, thereafter they received diets nos. 1785, 1787, 1789, and 1791, respectively.

However, the animals from the groups receiving pyridoxine-free diets all the time immediately from hatching (groups 1786 and 1790) died within 2 $^1/_2$—4 weeks. At the end of this period they had severe convulsions. Two corresponding (pyridoxine-free) groups, which were shifted to pyridoxine-supplemented diets (3.5 mg pyridoxine hydrochloride per 100 g of diet) after two weeks (groups 1788 and 1792), resumed growth and survived, but were not subjected to analysis because the pyridoxine-free control groups were missing.

The second section (B) only comprised four groups (groups 1837, 1838, 1839, 1840); all groups received the "normal" commercial diet (DAM *et al.* 1957) for two weeks, thereafter they were shifted to diets corresponding to nos. 1785, 1789, 1787 and 1791, respectively, but with 1 % cholesterol in all diets at the cost of the corresponding amount of sucrose.

At the end of the experimental feeding period (4 weeks), 4 ml blood was taken by means of a heparinized syringe from the jugular vein of each chick of the two sections. Thereafter, the chicks were sacrificed by decapitation, autopsied, and liver, heart, and aorta taken out for chemical determinations.

The 4 ml blood from each chick was transferred to a centrifuge tube containing two drops of heparin solution (one drop = 0.04 ml) and was centrifuged for 6 min at 3,000 rpm. The total content of plasma cholesterol was determined by the method of ABELL *et al.* (1952).

The organs were treated and analyzed for cholesterol and polyenoic fatty acids as described earlier (DAM *et al.* 1956) using the Liebermann-

[1] Containing only 0.1 % total fatty acids. The amount of polyenoic acids in the total fatty acids was determined by alkali isomerization. The following values were found: Dienoic, 16.3 %; trienoic, 2.4 %; tetraenoic, 0.5 %; pentaenoic, 0.4 %.

Table I.

Composition of diets, g per kg.

Diet no.	1785	1787	1789	1791
Casein, "Vitamin test"[1]	200	200	200	200
Gelatine	80	80	80	80
Salt mixture[2]	51.7	51.7	51.7	51.7
Vitamin mixture no. 4[3]	0	1.0	0	1.0
Vitamin mixture no. 7[4]	1.0	0	1.0	0
Choline chloride	1.5	1.5	1.5	1.5
Sucrose	665.8	665.8	565.8	565.8
Peanut oil[5]	0	0	100.0	100.0

All diets were supplemented with 10 mg Synkavit "Roche" (Di-calciumsalt of 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester) and 100 mg Ephynal "Roche" (d,l-α-tocopherol acetate) per kg. Vitamins A and D_3 were given as a solution prepared from crystalline vitamin A acetate (Roche), 1 g; crystalline vitamin D_3 (Roche), 0.0058 g; ethyl alcohol, 100 ml; "Tween 80", 64 g; and distilled water *ad* 330 ml, 0.1 ml twice a week per animal, furnishing 250 I. U. A and 20 I. U. D_3 per chick per day.

[1] From Genatosan Ltd., Loughborough, England; containing 0.11 % fat.
[2] Secondary calcium phosphate, 2 aq., 2,800 g; calcium carbonate, 875 g; desiccated magnesium sulphate, Ph. Dan., 404 g; potassium chloride, Ph. Dan., 460 g; sodium chloride, Ph. Dan., 500 g; ferric citrate (about 17.5 % Fe), 100 g; manganese sulphate (water-free), 23 g; cupric sulphate, 5 aq., Ph. Dan., 2 g; zink sulphate, 7 aq., Ph. Dan., 1 g; aluminium sulphate, 18 aq., Ph. Dan., 1 g; magnesium silicate, Ph. Dan., 1 g; diiodotyrosine, Ph. Dan., 1 g; cobolt carbonate, 0.05 g; total 5,168.05 g.
[3] Thiamine hydrochloride, 3 mg; riboflavin, 4 mg; nicotinic acid, 50 mg; calcium pantothenate, 12 mg; pyridoxine hydrochloride, 3.5 mg; biotin, 0.1 mg; folic acid, 2.0 mg; sucrose, 925.4 mg; total, 1,000.0 mg.
[4] Vitamin mixture no. 4 without pyridoxine.
[5] From A/S Dansk Sojakagefabrik, Copenhagen. Content of polyenoic fatty acids, determined by alkali-isomerization, showed the following values: Dienoic, 24.7 %; trienoic, 0.5 %; tetraenoic, 0.0 %; preformed conjugated dienoic, 0.4 %.

Burchard reaction for cholesterol determination in liver and heart, the Tschugaeff reaction (modified by HANEL and DAM 1955) for aorta, and the alkali-isomerization procedure of HAMMOND and LUNDBERG (1953) for the polyenoic acids (pentaenoic acid assumed to contain 22 carbon atoms).

Results and Discussion.

Growth.

The average body weights for each group, at weekly intervals, are shown in Fig. 1. As found by other investigators (LEPKOVSKY and KRATZER 1942, COMBRIDGE 1956), the growth was retarded or nearly stopped just after changing the diet from "normal" to pyridoxine-deficient. Thus, it is obvious that no reserve of the

Table I a.

Diets for the various groups of chicks.

Group no.	Section A								Section B			
	1785	1787	1789	1791	1786	1788	1790	1792	1837	1838	1839	1840
Diet fed during the first two weeks after hatching	"normal"				1785 (fat-free, pyridoxine-free)				"normal"			
Thereafter diet no.	1785	1787	1789	1791	1785	1787	1789	1791	1785 + 1 % cholesterol	1789	1787	1791

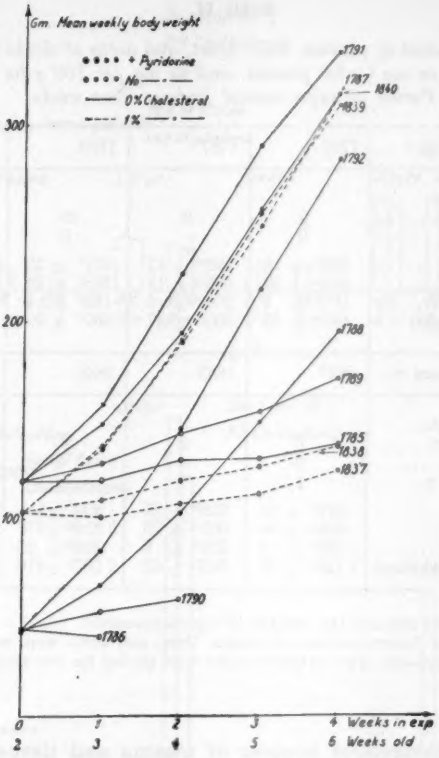

Fig. 1. Weight curves showing mean weekly body weight plotted against experimental weeks and age of the chicks. The figures at the end of each curve refer to group numbers.

vitamin is present in the bodies of the chicks. The growth curves for the chicks receiving dietary pyridoxine show a steady, normal weight gain during all four experimental weeks. Peanut oil plus pyridoxine gave the fastest growth. Addition of peanut oil to the pyridoxine-free diets gave only slight improvement of growth compared with the corresponding fat-free, pyridoxine-free diet.

Dietary cholesterol (1 %) (groups 1837, 1838, 1839, 1840) did not seem to influence growth.

Table II.

Cholesterol content of plasma, liver, heart, and aorta of chicks. The results are expressed as mg % for plasma, and as mg per 100 g for the tissues. Period of experimental feeding: four weeks.

Section A, groups no.	1785	1787	1789	1791
Addition to diet:				
Peanut oil, %	0	0	10	10
Pyridoxine	0	+	0	+
Plasma	$226^{9} \pm 14$	$159^{10} \pm 12$	$237^{7} \pm 15$	$152^{8} \pm 13$
Liver	$402^{9} \pm 36$	$386^{10} \pm 18$	$333^{8} \pm 23$	$311^{9} \pm 21$
Heart	$167^{9} \pm 1$	$200^{10} \pm 6$	$182^{8} \pm 4$	$185^{9} \pm 2$
Aorta (dry weight) ...	$687^{6} \pm 47$	$600^{10} \pm 27$	$689^{8} \pm 70$	$496^{9} \pm 39$
Section B, groups no.	1837	1839	1838	1840
Addition to diet:				
Peanut oil, %	0	0	10	10
Pyridoxine	0	+	0	+
Cholesterol, %	1	1	1	1
Plasma	$327^{11} \pm 20$	$218^{12} \pm 21$	$920^{11} \pm 111$	$596^{11} \pm 65$
Liver	$625^{11} \pm 39$	$567^{12} \pm 29$	$3\,359^{11} \pm 378$	$2\,914^{11} \pm 237$
Heart	$195^{11} \pm 5$	$233^{12} \pm 8$	$299^{11} \pm 23$	$368^{11} \pm 17$
Aorta (dry weight) ...	$1\,125^{11} \pm 87$	$885^{11} \pm 65$	$2\,037^{11} \pm 216$	$1\,698^{11} \pm 163$

The superscripts indicate the number of organs examined.
The cholesterol determinations of plasma, liver, and aorta were carried out for each chick separately. Two to three hearts were pooled for one analysis.

Cholesterol content of plasma and tissues (Table II, Fig. 2 and 3).

In both experiments, *plasma* cholesterol was significantly higher in the chicks fed pyridoxine-deficient diets than in the chicks receiving diets with added pyridoxine. This is in accordance with the effect of pyridoxine-deficiency on the plasma-cholesterol of Rhesus monkeys on a low fat diet containing 1 % cholesterol (GREENBERG and RINEHART 1951). The higher cholesterol values in section B are due to the addition of cholesterol to the diet.

Liver cholesterol was not significantly affected by the pyridoxine deficiency in these two experiments.

In both sections (A and B), the pyridoxine-deficient chicks had a somewhat higher cholesterol content in the *aorta* than the pyridoxine-fed chicks.

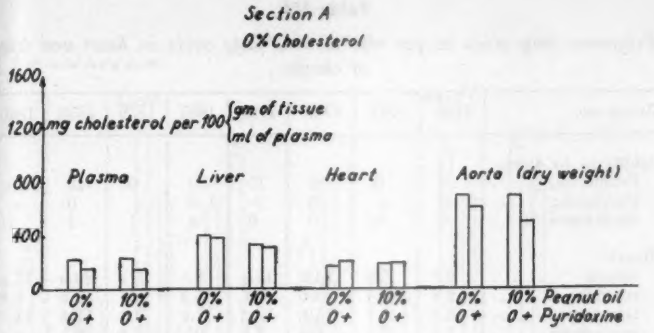

Fig. 2.

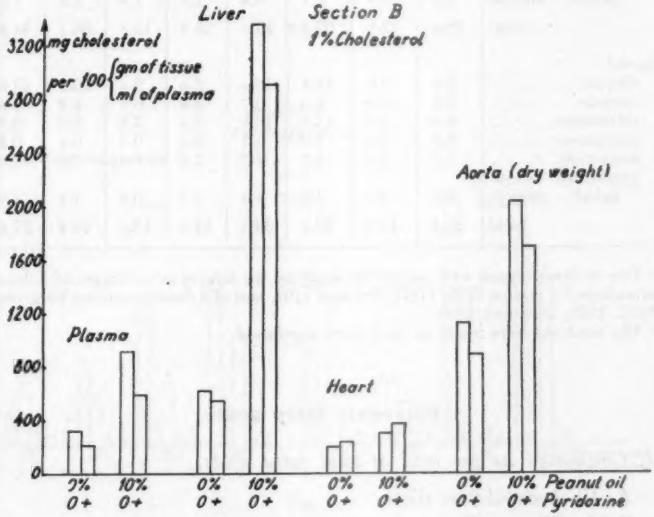

Fig. 3.

Fig. 2 and Fig. 3. Cholesterol content of plasma, liver, heart, and aorta of chicks fed 0 % or 10 % peanut oil and 0 or 1 % cholesterol with and without pyridoxine supplementation.

With regard to *heart* cholesterol, the diet *without* added pyridoxine caused lower values than did the corresponding diets *with* added pyridoxine, except for the peanut oil-fed groups in section A.

Table III.

Polyenoic fatty acids in per cent of total fatty acids in heart and liver of chicks.

Group no.	1785	1787	1789	1791	1837	1839	1838	1840
Additions to diets:								
Peanut oil, % ...	0	0	10	10	0	0	10	10
Pyridoxine	0	+	0	+	0	+	0	+
Cholesterol, % ...	0	0	0	0	1	1	1	1
Heart[1]								
dienoic	9.0	6.3	16.6	17.4	8.1	5.1	20.9	17.5
trienoic	2.1	6.5	0.1	—0.1	3.2	5.3	1.0	0.0
tetraenoic	14.0	4.9	13.4	15.3	11.0	3.6	12.3	14.3
pentaenoic	1.1	0.2	1.0	1.0	0.7	0.2	0.5	1.1
hexaenoic	0.5	0.2	0.6	0.5	0.3	0.0	0.1	0.5
preformed conjugated dienoic	1.7	0.9	1.7	0.6	1.0	1.6	1.3	1.5
total	28.4	19.0	33.4	34.7	24.3	15.8	36.1	34.9
Liver[2]								
dienoic	5.2	3.3	18.4	12.4	2.8	2.4	16.3	13.8
trienoic	3.8	6.9	1.9	0.7	3.2	6.2	1.3	1.4
tetraenoic	8.2	4.9	13.0	16.8	5.5	3.5	5.3	9.3
pentaenoic	0.8	0.4	1.3	1.7	0.5	0.2	0.3	0.6
hexaenoic	1.7	1.1	1.7	1.7	1.0	0.6	0.6	0.9
preformed conjugated dienoic	0.9	0.7	1.3	1.4	1.0	0.6	1.1	1.0
total	20.6	17.3	37.6	34.7	14.0	13.5	24.9	27.0

[1] Two or three organs were pooled for analysis; the figures are averages of 3 determinations for groups 1785, 1787, 1789 and 1791, and of 4 determinations for groups 1837, 1839, 1838 and 1840.
[2] The analyses were made on each liver separately.

Polyenoic fatty acids.

1. Calculated as per cent of total fatty acids.

A. Cholesterol-free diets.

Table III and Fig. 4 show that when the diets are fat-free the accumulation of 5,8,11-trienoic acid (eicosatrienoic acid (MEAD and SLATON 1956)) in liver and heart is much less when pyridoxine is lacking in the diet, whereas, under the same circumstances, the amounts of dienoic and especially tetraenoic are increased. Assuming that the conversion processes are as proposed by MEAD and SLATON: 9,12-dienoic $\rightleftharpoons$ 5,8,11,14-tetraenoic $\rightleftharpoons$ 5,8,11-eicosatrienoic acid, the present findings point to the necessity of pyridoxine for these reactions.

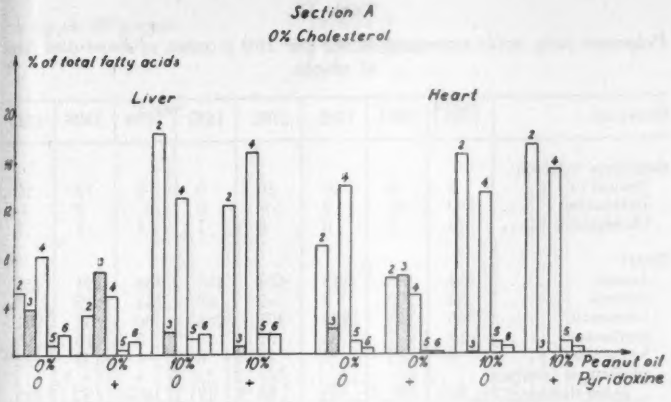

Fig. 4.

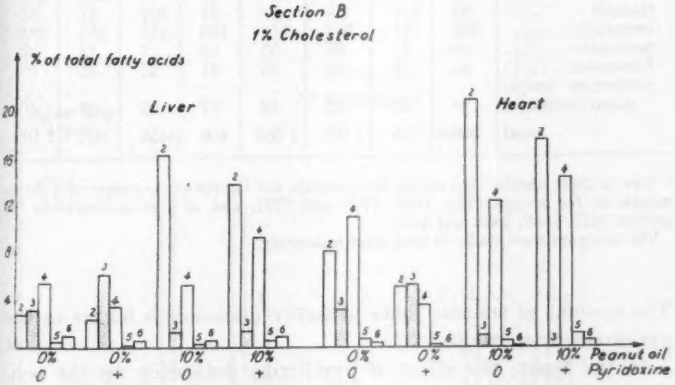

Fig. 5.

Fig. 4 and Fig. 5. Polyenoic fatty acids (expressed as % of total fatty acids) in liver and heart of chicks reared on fat-free or 10 % peanut oil-diets with and without 1 % cholesterol and pyridoxine supplementation.

The other polyenes are present in small amounts only.

When the diets contain 10 % of peanut oil the accumulation of tetraenoic acid in the liver is less when pyridoxine is lacking whereas the corresponding amount of dienoic is higher. This is in agreement with findings for rats (WITTEN and HOLMAN 1952).

Table IV.

Polyenoic fatty acids expressed as mg per 100 g organ of heart and liver of chicks.

Group no.	1785	1787	1789	1791	1837	1839	1838	1840
Additions to diets:								
Peanut oil, % ...	0	0	10	10	0	0	10	10
Pyridoxine	0	+	0	+	0	+	0	+
Cholesterol, % ...	0	0	0	0	1	1	1	1
Heart[1]								
dienoic	154	188	412	534	155	138	391	452
trienoic	36	192	2	—2	60	141	19	0
tetraenoic	240	145	329	471	208	96	231	371
pentaenoic	18	7	25	31	13	5	10	27
hexaenoic	8	7	14	16	6	1	2	12
preformed conjugated dienoic ..	30	26	43	20	17	40	25	37
total	486	565	825	1 070	459	421	678	899
Liver[2]								
dienoic	125	105	507	384	85	80	648	565
trienoic	93	214	55	24	91	207	51	56
tetraenoic	201	151	349	511	159	115	215	384
pentaenoic	19	11	33	50	13	7	13	26
hexaenoic	42	33	44	50	31	20	21	35
preformed conjugated dienoic ..	23	21	34	43	27	21	43	40
total	503	535	1 022	1 062	406	450	991	1 106

[1] Two or three organs were pooled for analysis; the figures are averages of 3 determinations for groups 1785, 1787, 1789 and 1791, and of 4 determinations for groups 1837, 1839, 1838 and 1840.

[2] The analyses were made on each liver separately.

The amount of trienoic (here probably linolenic) is higher in the pyridoxine-free groups.

In the heart, the effect of pyridoxine deficiency on the conversion of dienoic to tetraenoic is not marked; and trienoic is practically absent, irrespective of whether pyridoxine is given or not.

B. Diets containing 1 % cholesterol. Table III and Fig. 5.

The most pronounced difference from the results obtained with cholesterol-free diets is that when 10 % peanut oil is fed, liver tetraenoic acid is very much depressed by cholesterol addition. This has been found earlier for pyridoxine-containing diets (DAM *et al.* 1956, DAM and KOFOED NIELSEN 1956). The present experiments show that the difference is even more marked when cholester-

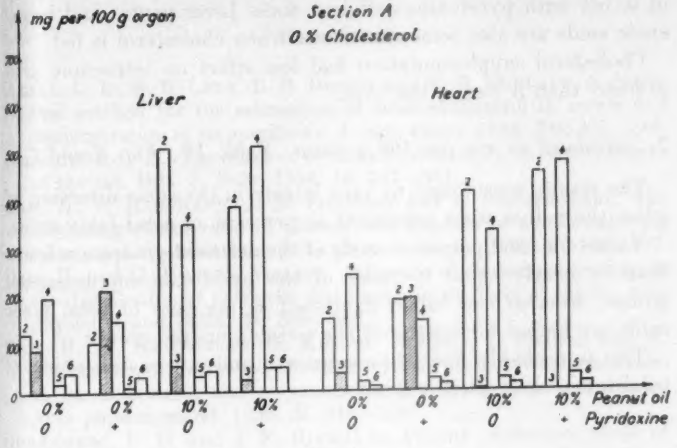

Fig. 6.

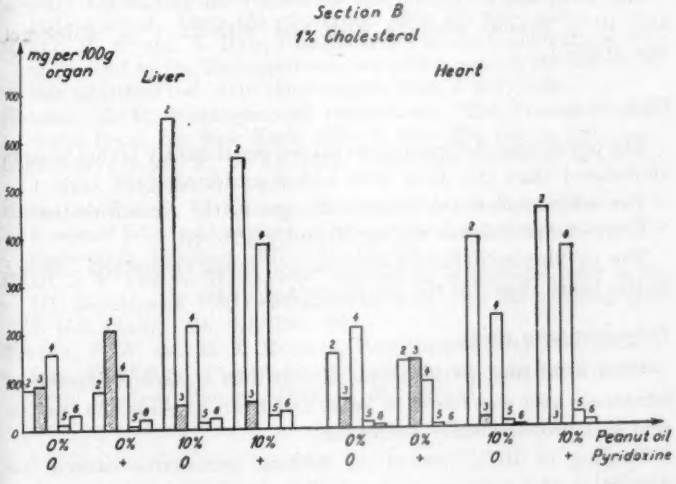

Fig. 7.

Fig. 6 and Fig. 7. Polyenoic fatty acids (expressed as mg/100 g tissue) in liver and heart of chicks reared on fat-free or 10 % peanut oil-diets with and without 1 % cholesterol and pyridoxine supplementation.

ol is fed with pyridoxine-deficient diets. Liver penta- and hexaenoic acids are also somewhat lower when cholesterol is fed.

Cholesterol supplementation had less effect on tetraenoic acid in heart than it had in the liver.

2. Calculated as mg per 100 g tissue. Table IV, Fig. 6 and 7.

The results were found to vary largely in the same direction as when the values were calculated as per cent of total fatty acids.

Values for total polyenoic acids of the deficient groups are lower than or nearly equal to those of the pyridoxine-supplemented groups, whereas the values expressed as per cent of total fatty acids are higher for nearly all the pyridoxine-free groups.

This is probably due to poor growth and scarce deposition of total fat in the deficient groups.

Summary.

The influence of pyridoxine deficiency on chicks fed fat-free and 10 % peanut oil-diets with and without 1 % cholesterol was studied.

Cholesterol.

The pyridoxine-deficient diets caused significantly higher plasma cholesterol than the diets with added pyridoxine.

The aorta cholesterol showed changes in the same direction.

Liver cholesterol was not significantly affected.

The pyridoxine-deficient diets caused lower cholesterol content in the heart than did the supplemented diets.

Polyenoic fatty acids.

With a fat-free, pyridoxine-deficient diet a marked content of tetraenoic acid was found in heart and liver, whereas the trienoic acid was proportionally decreased.

Feeding of 10 % peanut oil without pyridoxine caused less deposition of tetraenoic in heart and especially in the liver than feeding of 10 % peanut oil with pyridoxine.

Addition of 1 % cholesterol to the diets resulted in a decrease in the amount of tetraenoic acid in heart and liver.

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The Life Span of the Erythrocytes of Normal and of Tumour-bearing Mice as Determined by Glycine-2-^{14}C.

By

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The fundamental abnormality leading to the development of anaemia in patients with neoplastic disease is a marked shortening of the life span of the erythrocytes (BERLIN, 1951, BERLIN, LAWRENCE and LEE 1951, 1954, ROSS, CROCKETT and EMERSON 1951, HYMAN 1954, 1958, SHEETS *et al.* 1954, HYMAN and HARVEY 1955, BERLIN and LAWRENCE 1956, ROSS and MILLER 1956, HYMAN, GELLHORN and HARVEY 1956, MILLER *et al.* 1956, DAL SANTO 1957, SOHIER, JURANIES and AUB 1957).

The question why red corpuscle survival should be shortened in patients with neoplastic disease is unanswered. Certain important facts relating to the problem are however known.

By employing radiochromium tagged erythrocytes and the Ashby differential agglutination technique, it has been possible to show that both normal red corpuscles transfused into patients suffering from cancer, as well as the patient's own erythrocytes have a similar decreased longevity in the circulation of the patient (HYMAN and HARVEY 1955, ROSS and MILLER 1956, MILLER *et al.* 1956, HYMAN *et al.* 1956, HYMAN 1958).

Transfusion of whole blood from patients with advanced neo-

plastic disease into healthy volunteers revealed an essentially normal life span in 11 of 18 instances (HYMAN 1958).

By retransfusion into normal subjects of normal red corpuscles which had remained in the circulation of cancer patients for variable periods of time, it was shown that exposure of these erythrocytes to the influence of the "neoplastic milieu", for periods as brief as 7 days, resulted in irreparable changes in the erythrocytes and consequent shortening of the life span (ROSS and MILLER 1956). This indicates that the shortened survival of the erythrocytes in neoplastic disease is probably due to an extracorpuscular influence (ROSS and MILLER 1956, HYMAN *et al.* 1956, HYMAN 1954, 1958).

In the present communication we report studies on the life span of the erythrocytes of normal and of tumour-bearing mice, applying a method that was introduced by SHEMIN and RITTENBERG (1946) for the measurement of the life span of the erythrocytes of man and chicken (SHEMIN 1948) with ^{15}N-labelled glycine and which subsequently has been used to determine the red corpuscle life span of the dog (GRINSTEIN, KAMEN and MOORE 1949), rat (BERLIN, MEYER and LAZARUS 1951), cat (VALENTINE *et al.* 1951), rabbit (NEUBERGER 1951), man (BERLIN *et al.* 1951, 1954), marmot (BRACE 1953), pig (BUSCH *et al.* 1955), and duck (BRACE and ALTLAND 1956) with glycine labelled with ^{14}C.

It will be shown that the life span of the erythrocytes of cancerous mice is shortened and that this is partly due to an extracorpuscular influence and probably also partly due to an intrinsic defect of the red corpuscles formed in the cancerous organism.

Material and Methods.

Male TE mice, kindly supplied by the Animal Department of the British Medical Council, were injected with Ehrlich ascites carcinoma cells suspended in saline.[1] Each animal received 2—6 × 10^6 cells, in 0.1 ml saline, intramuscularly into the right hind leg. Progressive tumour growth occurred in all animals. The average body weight of the animals was 32 g; they were kept on a standard diet.

Female C3H mice, about 6 months old, all having spontaneous mammary carcinoma, were used in one experiment. In order to

[1] Thanks are due to Prof. G. KLEIN for kindly supplying us with the Ehrlich ascites carcinoma and also with the C3H mice.

obtain controls of this strain, we were obliged to take C3H males of the same age and body weight. The animals weighed 25—30 g.

Glycine, labelled with ^{14}C in the α-carbon atom, of a specific activity of 1 millicurie per 15.6 mg glycine was used. 2 microcuries dissolved in 0.1 ml saline were injected intraperitoneally into each animal.

Groups of five animals were killed at different times after the injection of the labelled glycine. The mice were bled from the retroocular sinus under Nembutal narcosis. Heparin was used as an anticoagulant.

The blood of five animals was centrifuged, the supernatant plasma removed, and the red corpuscles washed three times with saline.

The haemin was isolated and recrystallized to constant specific activity, as previously described by FISCHER (1941) and SHEMIN *et al.* (1950). Two recrystallizations were found to be sufficient to give samples of constant specific activity.

The dry haemin crystals were suspended in ether and pipetted into weighed standard aluminium dishes (1.4 cm diam.). The ether was then evaporated, and the weight of the dry haemin crystals determined.

The radioactivity of the samples was measured in an end window Geiger Müller counter (window thickness 1.9 mg per cm^2). If necessary, appropriate corrections for self-absorption to infinite thickness were made. The error of the counting and correcting procedure was $\pm$ 3 per cent.

Results.

In the first experiment, 85 male TE mice were injected intraperitoneally with glycine-2-^{14}C. One group of 5 animals was killed on the fifth day. On the same day Ehrlich ascites carcinoma was inoculated into 35 of the glycine-injected mice. Groups of 5 mice, both of the cancer and control series, were killed in the beginning at approximately 5-day intervals and later at about 10-day intervals. The haemin was isolated from the blood and measured as described above. Deaths occurred spontaneously in the cancerous mice on the 40th day after tumour inoculation; no later groups of cancerous animals could therefore be analyzed.

Fig. 1 shows the specific activity of the haemin in counts/min/-

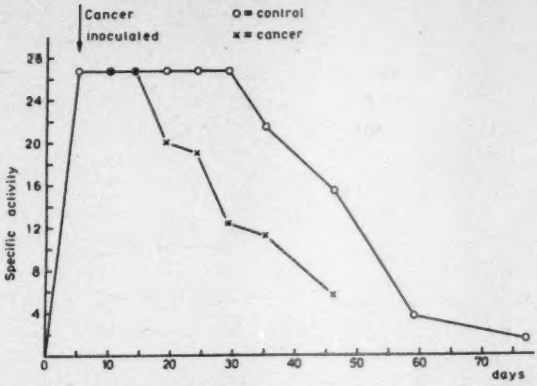

Fig. 1. Specific activity of the haemin of cancerous and control TE mice in counts/min/mg haemin as a function of time after intraperitoneal injection of glycine-2-^{14}C. Ehrlich ascites carcinoma was inoculated intramuscularly on the fifth day after administration of the tracer.

mg haemin as a function of time after administration of the ^{14}C labelled glycine in cancerous and control mice. It is seen that as early as approximately ten days after tumour inoculation, the amount of ^{14}C in the red corpuscles of the cancerous mice begins decreasing. Analysis by the method of SHEMIN and RITTENBERG (1946) shows that the mean life span of the red blood corpuscles of mice, inoculated with Ehrlich ascites carcinoma five days after the administration of the glycine-2-^{14}C, is 26 days. The control mice had a mean red corpuscle life span of 46 days.

In five other experiments with normal male TE mice, the mean red corpuscle life span as calculated by the method of SHEMIN and RITTENBERG (1946) varied from 40—43 days, the mean value being 41 days.

In a second experiment we injected 40 male TE mice intramuscularly with 2×10^6 cells of the Ehrlich ascites carcinoma. Ten days after the tumour inoculation the cancerous and 50 healthy control mice were injected intraperitoneally with glycine-2-^{14}C. Groups of 5 animals of both the cancer and control series were killed at the third and at the sixth day, and then at approximately 5-day intervals. The specific activity of their blood haemin was determined. The cancerous animals died spontaneously after

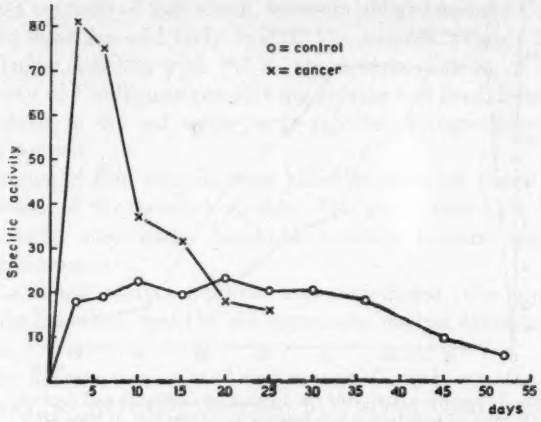

Fig. 2. Specific activity of the haemin of cancerous and control TE mice in counts/min/mg haemin as a function of time after intraperitoneal injection of glycine-2-^{14}C. Ehrlich ascites carcinoma was inoculated intramuscularly ten days prior to the administration of the tracer.

the 35th day after tumour inoculation, no later groups could therefore be obtained.

Fig. 2 shows the specific activity of the haemin as a function of time in cancerous and control mice. It is seen that, in the tumour bearing mice, the specific activity reaches a maximum on the 3rd day and then, in contrast to the controls, falls rapidly.

A direct plot of the specific activity of the haemin after the 3rd day in a semilogarithmic coordinate system gives, within the experimental error, a straight line with a half time of 10 days. This curve results from a random destruction of the red blood corpuscles (LONDON *et al.* 1949). Since the red blood corpuscles, formed in the cancerous organism are destroyed in an indiscriminate fashion rather than as a function of their age, their mean survival time is given by the expression (LONDON *et al.* 1949):

$$\bar{T} = \frac{t_{\frac{1}{2}}}{\ln 2}$$

Their mean survival time $\bar{T}$ is therefore equal to 14 days.

Fig. 2 also shows that about 4 times more ^{14}C is incorporated

into the haemin of the erythrocytes of the cancerous animals, than into the haemin of the erythrocytes of the controls.

The control mice in this experiment had a mean erythrocyte life span of 43 days.

In another experiment, 20 female C3H mice, all having spontaneous mammary carcinoma, and 30 healthy male C3H mice, were injected intraperitoneally with glycine-2-^{14}C. Groups of five animals were killed at different times after the glycine injection. The results of this experiment were essentially the same as in the experiment with the TE mice inoculated intramuscularly with Ehrlich ascites carcinoma ten days prior to the administration of the glycine-2-^{14}C (cf. Fig. 2).

The specific activity of the haemin in C3H mice bearing spontaneous mammary carcinoma rises to a maximum which is 3 to 4 times higher than in the controls and then declines rapidly following the pattern of a single exponential process with a half time of 11 days, indicating a mean survival time of 16 days for the erythrocytes in the female C3H mice bearing spontaneous mammary carcinoma.

The male C3H control mice had a mean red corpuscle life span of 40 days.

Discussion.

The finding of a mean erythrocyte life span of 42 days in normal mice is in contrast to the findings of previous investigators using radioactive iron (25 days) (Burwell, Brickley and Finch 1953) and sulphhaemoglobin (19 days) (Dod, Bierman and Shimkin 1951). Preference must be given, however, to the physiological tagging of the erythrocytes with labelled agents which are not reutilized, *e. g.* glycine labelled with ^{15}N or ^{14}C (Berlin, Lawrence and Elmlinger 1957, Berlin *et al.* 1957).

It is shown that the erythrocytes of normal mice are not destroyed at random but that their rate of destruction is a function of their age, as has previously been found for the erythrocytes of man (Shemin and Rittenberg 1946), dog (Grinstein *et al.* 1949, Bale *et al.* 1949), rat (Berlin *et al.* 1951) and chicken (Ottesen 1955).

The average life span $\bar{T}$, is related to the rate at which red blood

corpuscles are delivered to the circulation. This relationship is given by the equation (LONDON *et al.* 1949):

$$\overline{T} = \frac{M}{m}$$

in which M is the total circulating red corpuscle haemoglobin and m is the mass of red corpuscle haemoglobin delivered to the circulation per day. This relationship holds only when M is constant; *i. e.*, the rate of synthesis of red corpuscle haemoglobin is equal to the rate of its destruction.

It has been shown by the author (1958) that the total circulating haemoglobin in the healthy male TE mice studied was 305 mg per animal and that it remained constant during the course of the investigation. Since the mean life span of the erythrocytes was 42 days, the rate of haemoglobin production and destruction was 7.3 mg of haemoglobin per animal per day. With a mean circulating red corpuscle mass of 0.83 ml per animal, the rate of red corpuscle production and destruction is 0.02 ml per animal per day.

The finding that the life span of normal erythrocytes, formed in a healthy organism, is shortened after a malignant tumour has been transplanted, confirms the earlier hypotheses that the shortened erythrocyte survival in patients with neoplastic disease is due to an extracorpuscular influence (ROSS and MILLER 1956, HYMAN *et al.* 1956, HYMAN 1954, 1958).

The nature of this extracorpuscular influence is at present unknown; some mechanisms, however, have been demonstrated to be present in the neoplastic organisms which affect the erythrocytes and probably render them more susceptible to destruction (WEIL 1907, GROSS 1947, 1949, HYMAN 1954, BROMBERG and ERDREICH 1956, BALDWIN 1957, CROSBY and BENJAMIN 1957, REID *et al.* 1957, ULTMANN *et al.* 1957, LOCKNER 1958). A probability also exists that hypersplenism is at least partly responsible for the shortened life span of the red blood corpuscles in the cancerous organism (BERLIN and LAWRENCE 1956, LAWRENCE 1956, ULTMANN 1958, v. EHRENSTEIN 1958). Studies on this problem are in progress in this laboratory.

The red blood corpuscles formed under the influence of a transplanted or spontaneous carcinoma have a mean survival time of only about 15 days and their pattern of destruction changes from destruction as a function of age to random destruction.

This pattern of erythrocyte destruction has also been found in patients with chronic lymphatic leukaemia when shortening of the erythrocyte life span was observed (BERLIN and LAWRENCE 1956).

Our results compare well with the finding of DOD *et al.* (1951), who found that the life span of the erythrocytes, measured by the sulphhaemoglobin method, in male C3H mice bearing transplanted mammary carcinoma is only about half that of healthy control mice.

We are inclined to the view that the short survival time and the random destruction of the red corpuscles in the cancerous animals is not only due to an extracorpuscular influence, but that some intrinsic defect may also be present in the erythrocytes formed in the cancerous organism in response to increased haemolysis.

It is interesting in this connection to recall the findings of NEUBERGER and NIVEN (1951), BERLIN and LOTZ (1951) and of FRYERS and BERLIN (1952), who found that some of the red blood corpuscles produced at the outset of a period of rapid erythropoiesis due to haemorrhage (NEUBERGER and NIVEN 1951, BERLIN and LOTZ 1951) or due to hypoxia (FRYERS and BERLIN 1952) had an abnormally shortened survival time and a random destruction pattern. These findings were confirmed recently by LINMAN and LONG (1958) who found two distinct types of erythrocytes: microcytes with increased osmotic fragility and normal red corpuscles, in experiments with rats receiving the thermostable erythropoietic factor from "anaemic" rabbit plasma.

No definite conclusions could be drawn from our present experiments regarding the presence of more than one population of red corpuscles in the cancerous mice. Studies are in progress in this laboratory to elucidate this problem.

The total circulating haemoglobin in the cancerous mice was found by the author (1958) to be 314 mg per animal and was also found to be constant until a few days before death, when the tumour was ulcerated and bleeding occurred. The rate of haemoglobin production based on a mean red corpuscle survival time of 15 days was 21 mg of haemoglobin per animal per day. With a circulating red corpuscle mass of 0.90 ml per animal (v. EHRENSTEIN 1958), the red corpuscle production rate was 0.06 ml per animal per day. These rates of haemoglobin and red corpuscle production are about 3 times the normal. Since the total circulating haemoglobin and the total circulating red corpuscle mass re-

mained at the same level during the course of the study, the mice were also destroying red corpuscles at a rate 3 times the normal. The very rapid formation of erythrocytes must be accompanied by a hyperplasia of the blood-forming tissues. This hyperplasia is demonstrated by the finding that about 4 times more ^{14}C than normal is incorporated into the haemoglobin of the red corpuscles of the cancerous animals.

Summary.

1. The life span of the erythrocytes of healthy and of tumour-bearing mice was determined with glycine-2-^{14}C as a tracer.

2. The rate of destruction of the erythrocytes of healthy mice was found to be a function of the age of the erythrocytes.

3. The average life span of the erythrocytes of healthy male TE mice varied between 40—46 days, the mean value was 42 days. The corresponding figure for healthy male C3H mice was 40 days.

4. After the transplantation of an Ehrlich ascites carcinoma growing in the solid form, the average life span of the erythrocytes formed in healthy male TE mice was shortened to 26 days.

5. The erythrocytes of cancerous mice were found to be destroyed at random. Their mean survival time was 14 days in male TE mice bearing Ehrlich ascites carcinoma growing in the solid form and 16 days in female C3H mice bearing spontaneous mammary carcinoma. The rates of haemoglobin and red corpuscle formation and destruction were about 3 times the rates in healthy mice.

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A Study of Rumination Induced by Milking in the Goat.

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The fact that milking very often induces rumination in cows and goats is well known to most people handling these animals. No previous study of the phenomenon seems however to have been reported. For this reason, and as the reflex as such offers the possibility to induce and study rumination repeatedly at any time in one and the same animal, the following experiments were undertaken.

Methods.

Three lactating goats were selected for the experiments. The animals were supplied with closed ruminal, reticular and abomasal fistulae. The ruminal and abomasal fistulae were made principally as described by DOUGHERTY (1955). The reticular cannula was placed in position by means of a metal stylet (Fig. 1). The stylet, with the cannula around its central end, was introduced into the lumen of the reticulum via the rumen. It was then forced through the reticular and abdominal walls to emerge about 2 cm below the arcus costarum at the level of the 7th rib. The stylet was then removed and the cannula was fixed in the same manner as the ruminal and abomasal cannulae.

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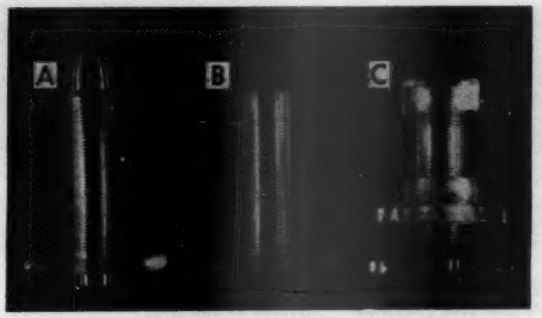

Fig. 1. A: A reticular, perspex cannula enclosing a metal stylet, which made it possible to force the cannula through the reticular and abdominal walls.
B: The cannula after removal of the stylet.
C: The closed cannula supplied with rings used to keep it in position in the abdominal wall.

Pressure recording: Pressure changes in the lumens of the dorsal sac of the rumen, the reticulum and the pars pyloricus of the abomasum were recorded by means of three airfilled rubber balloons (3 cm in diameter) each connected to a pressure transducer. Each transducer was connected to one channel of a four channel inkwriting ECG-apparatus. The fourth channel was used for recording the chewing movements by help of a subcutaneously applied surgical-needle electrode over the masseter muscle. During the recording of gastric motility the animals were tied in a similar manner, and in a similar kind of stall, as they regularly were between the experiments.

Results.

A. The general behaviour of goats during milking.

The effect of milking on the behaviour of goats has been studied in a large number of animals. Most lactating goats seem to like being milked as indicated by the fact that they stand still and calm when the teats are approached, and these animals generally start to ruminate soon after milking is commenced. If hungry and fed in connection with milking they may, however, turn their attention towards the food offered and start to eat. A minority of animals, which seem irritated by the act of milking or are distressed

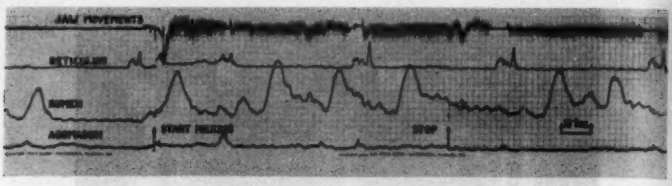

Fig. 2. An electromyogram of the masseter muscle (upper tracing) and simultaneous recordings of pressure changes (reading from top to bottom) in the reticulum, rumen (dorsal sac) and abomasum (pars pyloricus). Milking, which induced rumination, was performed during the period marked by the arrows.

Horizontal axis, 5 divisions: 10 sec.
Vertical axis, 1 division: Rumen 25 mm H_2O.
Reticulum and abomasum 50 mm H_2O.

by the conditions under which milking is performed, generally do not ruminate when milked.

B. Experimental studies of the milking-rumination reflex.

If the animals were calm, milking generally stimulated them to ruminate within 2—3 seconds after touching the teats (Fig. 2). The latency of the reflex was not related to the time of prior contraction of the reticulum as rumination could be elicited at varying intervals during the reticulo-ruminal cycle without influencing the latency. The duration of the first rumination cycle (regurgitation, chewing and swallowing) was usually shorter than the subsequent cycles. The cycles would continue at regular intervals during the entire period of milking even when no milk was obtained by squeezing the teats. Cessation of milking usually was followed by a cessation of rumination within a minute, although spontaneous rumination sometimes continued longer. If milking was stopped during the chewing phase of a rumination cycle and spontaneous rumination was not continuing after cessation of milking, the last chewing phase was often considerably prolonged.

It was observed at the beginning of the series of experiments that merely touching the teats or massage of the udder was in one of the animals sufficient to induce rumination. The sensitive area of the udder seemed to be limited to the part innervated by the lumbar nerves as touching the posterior, dorsal part of the udder, innervated by the superficial perineal nerve, and sensory stimulations of other areas adjacent to the udder did not induce rumination. However, when the goat had been used for experiments of this

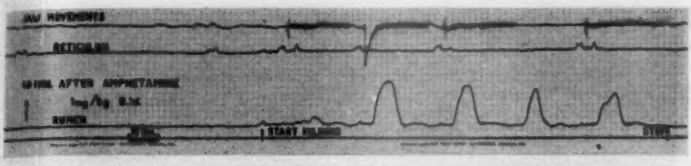

Fig. 3. An electromyogram of the masseter muscle (upper tracing) and simultaneous recordings of pressure changes in the reticulum and rumen (dorsal sac) started 40 min after intramuscular administration of amphetamine (1 mg/kg body weight). Milking which induced rumination was performed during the period marked by the arrows.

Horizontal axis, 5 divisions: 10 sec.
Vertical axis, 1 division: Reticulum 50 mm H_2O.
Rumen 25 mm H_2O.

kind during a month the milking situation had developed into a conditioned stimulus sufficient to elicit rumination, providing the goat was calm and expected to be milked. Thus when the animal watched the milker's slow approach to her back she might start to ruminate before the milker had actually touched her. Under this circumstance rumination was seen to commence immediately before a regular reticular contraction.

Milking or udder massage as a rule markedly increased the frequency and often also the strength of reticular and ruminal contractions. That happened even when milking was started during a period of spontaneous rumination. On a few occasions an increase in amplitude of abomasal contractions was also seen, although this was generally not the case. The influence of milking or udder massage on the motility of the forestomachs was much accentuated when the goats were under influence of amphetamine. Amphetamine (1—1.5 mg per kg b.w.) given intramuscularly caused an almost complete inhibition of ruminal motility within a few minutes and gave a marked reduction of the frequency of the reticular contractions. Milking performed 30 minutes after the administration of the drug did, however, still induce rumination and during the period of milking, strong ruminal contractions reappeared and the frequency of the reticular contractions increased (Fig. 3).

As could perhaps be expected from previous work showing that atropine does not prevent rumination (Wester 1923, Duncan 1954) it was found that this drug, although administered in doses causing a complete inhibition of stomach motility, did not block the milking— umination reflex. Fig. 4 shows a record of rumina-

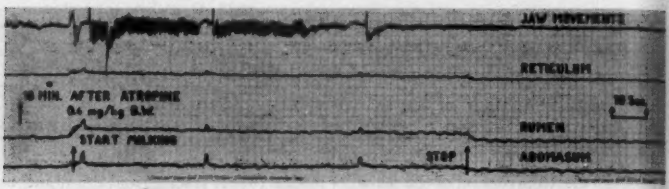

Fig. 4. An electromyogram of the masseter muscle (upper tracing) and simultaneous recordings of pressure changes (reading from top to bottom) in the reticulum, rumen (dorsal sac) and abomasum (pars pyloricus) started 10 min after the intramuscular administration of atropine (0.4 mg/kg b.w.). Milking which induced rumination but no stomach motility was performed during the period marked by the arrows.

Horizontal axis, 5 divisions: 10 sec.
Vertical axis, 1 division: Rumen 25 H_2O.
Reticul um and abomasum 5 mm $0H_2O$.

tion induced by milking in an atropinized goat (given 0.4 mg of atropine sulphate per kg b.w. intravenously 10 minutes prior to milking).

ARDUINI and DAGNINO (1953) have reported that large doses of apomorphine cause hyperkinesis of the rumen and forced mastication in the sheep. This, however, does not seem to be the case in the goat. Thus intravenous injections of relatively small doses of apomorphine hydrochloride (0.04 mg per kg b.w.) caused a complete inhibition of the reticulo-ruminal motility lasting for about 20 minutes followed by a gradual reappearance of reticular and ruminal contractions. Forceful, rhythmic, abomasal contractions started within three minutes after the intravenous injection of such a dose apomorphine and continued during the entire period of observation (up to 30 minutes). When milking was performed during the period of complete inhibition of reticulo-ruminal motility it still induced rumination and a temporary reappearance of ruminal contractions (Fig. 5).

Large doses of apomorphine (0.3 mg per kg b.w.) given intramuscularly also caused a complete inhibition of reticulo-ruminal motility which was still present 40 minutes after administration of the drug, but no increased abomasal motility was observed under these circumstances. When milking now was performed 10 to 20 minutes after the administration of the drug it no longer induced rumination but after a few seconds caused an intense licking which often continued for some time after milking was stopped (Fig. 6).

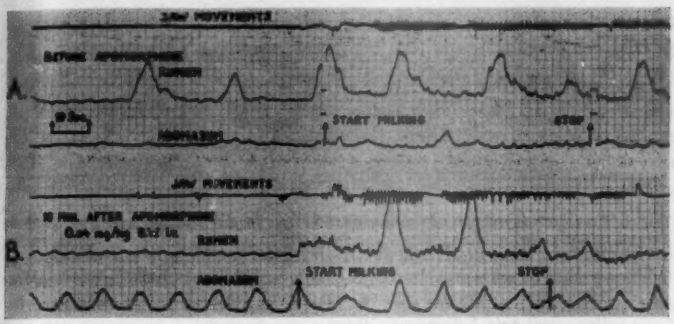

Fig. 5. A: An electromyogram of the masseter muscle (upper tracing) and simultaneous recordings of pressure changes in the rumen (dorsal sac) and the abomasum. Milking which induced rumination was performed during the period marked by the arrows.

B: A similar recording made 10 min after the intravenous administration of apomorphine (0.04 mg/kg b.w.).

Horizontal axis, 5 divisions: 10 sec.
Vertical axis, 1 division: Rumen 25 mm H_2O.
Abomasum 50 mm H_2O.

Discussion.

Present knowledge of the central regulation of rumination and of rhythmic reticulo-ruminal motility is too limited to allow a valid interpretation of the results reported here. It is known that the removal of a large part of the cerebral cortex or frontal lobectomy does not disturb reticulo-ruminal motility or prevent rumination in sheep and goats (Clark 1953, Bell and Lawn 1957). Clark (1953) has presented evidence to support the hypothesis that a subcortical area anterior to the pituitary infundibulum is intimately concerned with rumination. A few experiments have also been reported in which stimulations laterally in the anterior hypothalamus of unanaesthetized sheep and goats did change the behaviour of the animals in a particular way. They became calm and drowsy and started to ruminate after some minutes (Andersson 1951 b, Larsson 1954). But even if the act of rumination might be controlled from a supramedullary level it seems likely that the coordination of the sequence of events occurring during the rumination cycle is exerted by the medulla oblongata. It is at least obvious from the experiments of Titchen (1953) and of Iggo (1956) that sheep decerebrated by section in the intercollicular plane through

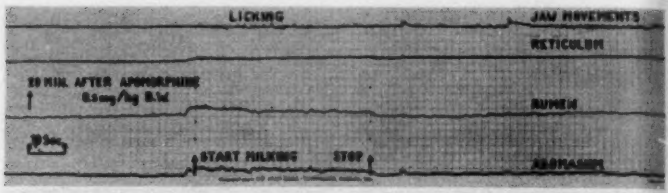

Fig. 6. An electromyogram of the masseter muscle (upper tracing) and simultaneous recordings of pressure changes (reading from top to bottom) in the reticulum, rumen (dorsal sac) and abomasum (pars pyloricus) started 20 min after the intramuscular administration of apomorphine (0.3 mg/kg b.w.). Milking which induced intense licking was performed during the period marked by the arrows.

Horizontal axis, 5 divisions: 10 sec.
Vertical axis, 1 division: Rumen 25 mm H_2O.
Reticulum and abomasum 50 mm H_2O.

the midbrain often retain spontaneous rhythmic reticulo-ruminal contractions.

Mechanical stimulation of the mucosa of the reticulum and rumen may induce rumination (SCHALK and AMADON 1928, and others). There is abundant evidence that the rumination reflex is stimulated by the contact of coarse roughage with the mucosa of the forestomachs (CLARK 1950, BALCH 1952) and it is probable that the desire to ruminate is related to augmented contractions of the reticulum and anterior part of the rumen induced by the presence of rough ingesta in the cardiac region (DUKES 1955). In the present study the milking stimulus was often seen to cause an increase of ruminal and reticular motility concomitant with rumination, but, as rumination started almost immediately and before any influence on the motility of the forestomachs was observed (Fig. 2), it suggests that the milking stimulus in itself can activate areas of the central nervous system concerned with rumination.

A comparison of the neuro-hormonal reflex of milk-ejection with the milking-rumination reflex reveals several similarities. Strong evidence has been presented to support the theory of ELY and PETERSEN (1941) that milking or suckling gives rise to nervous impulses which pass from the mammary gland via the anterior hypothalamus to the neurohypophysis and there cause a release of oxytocin (CROSS and HARRIS 1950, ANDERSSON 1951 a, b). When this hormone reaches the mammary gland it induces a contraction of the myoepithelial cells surrounding the alveoli.

It is well known that the milk-ejection reflex in the cow might readily become conditioned. Auditory and visual stimuli associated with the milking situation act as the conditioned stimuli. Milk-ejection has also been conditioned experimentally (Gračev 1952). It is further known that fright and discomfort can inhibit the milk-ejection reflex (Ely and Petersen 1941). Cross (1955) has shown in the rabbit that the main factor in this emotional inhibition is a central block of the release of oxytocin.

In one of the goats the milking situation obviously developed into a conditioned stimulus which in itself was sufficient to elicit rumination. The fact that irritated and restless animals do not ruminate in connection with milking indicates that the milking-rumination reflex might be emotionally inhibited in the same way as the milk-ejection reflex. A possible explanation to the above mentioned similarities between the milking-rumination and milk-ejection reflexes might be that the milking stimulus activates a "centre" (located in the anterior hypothalamus or in some other part of the brain stem) which is concerned both with the release of oxytocin from the neurohypophysis and with the development of the urge to ruminate. Irritation and discomfort might block this activation.

Amphetamine seems to exert an inhibitory action on hypothalamic "alimentary centres". Thus, evidence has been presented that this drug depresses food and water intake by an indirect inhibition of the hypothalamic "feeding and drinking centres" acting via the prefrontal and perhaps also other association areas of the cerebral cortex (di Ferrante and Longo 1953, Andersson and Larsson 1956). It seems to be possible to counteract the inhibition by the direct electrical stimulation of these "centres" (Andersson, Jewell and Larsson 1958). Amphetamine was here found to inhibit the reticulo-ruminal motility, but during milking this motility was reinforced (Fig. 3). It might therefore be assumed that the milking stimulus counteracts the inhibitory effect of amphetamine by a direct activation of a "centre" concerned with the regulation of reticulo-ruminal motility.

Borison and Wang (1949) have found that the vomiting centre is located to the lateral reticular formation of the medulla in the immediate vicinity of the fasciculus solitarius. As result of further studies on the central regulation of emesis the same authors (1951) have been able to show that apomorphine does not exert its emetic action by a direct stimulation of the vomiting centre.

The receptor site of apomorphine is situated outside the vomiting centre in the area postrema ("emetic chemoreceptor trigger zone").

Herbivorous animals and rodents seldom or never vomit and it has been suggested that this trait might be associated with some property of either the brain stem reticular formation or the area postrema in these animals (BORISON and WANG 1953). ARDUINI and DAGNINO (1953) studied the effect of large doses of apomorphine in the sheep and found that the drug caused hyperkinesis in the rumen and forced mastication. On the basis of this observation they suggested that the centre of rumination and that of emesis are identical and that accordingly rumination should be a particular form of vomiting. The only alimentary effects of small doses of apomorphine seen in the goat were rather forceful, rhythmic, abomasal contractions and an inhibition of reticulo-ruminal motility (Fig. 5). Mastication or emesis was not observed and the milking-rumination reflex was still present. Large doses of apomorphine caused an even more pronounced inhibition of reticulo-ruminal motility and in addition a disturbance of the milking-rumination reflex in such way that milking instead of rumination elicited intense licking (Fig. 6). Large doses of apomorphine have been shown to exert an anti-emetic action and evidence has been presented which indicates that this action is the result of a direct influence of the drug on the reticular formation (BORISON and WANG 1953). Accordingly, if the sequence of events occurring during the rumination cycle is coordinated in this part of the medulla oblongata the effect of large doses of apomorphine on the milking-rumination reflex might be the result of a disturbance of that coordination.

It has recently been observed that electrical stimulation of the lateral reticular formation of the medulla in the vicinity of the tractus solitarius elicits real vomiting in the unanaesthetized goat and that vomiting might be elicited by the application of copper sulfate solution in the abomasum (ANDERSSON, KITCHELL and PERSSON 1958). These observations taken together with the above mentioned effects of apomorphine in the goat strongly suggest that emesis and rumination involve two separate mechanisms.

Summary.

1. Milking is frequently seen to elicit rumination in cows and goats. The nature of this reflex was studied experimentally in lactating goats. Milking generally induced rumination within a few seconds and caused an increase of reticulo-ruminal motility. Similar to what has previously been observed for the milk-ejection reflex it was found that the milking situation could develop into a conditioned stimulus which in itself was sufficient to elicit rumination, and further that discomfort and irritation inhibited the milking-rumination reflex.

2. Amphetamine, atropine and small doses of apomorphine did not block the milking-rumination reflex. Milking performed after the administration of large doses of apomorphine, however, no longer induced rumination but caused intense licking. The observations made are discussed in the light of present knowledge of central regulation of rumination and of reticulo-ruminal motility.

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